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Combined effects of increased temperature and exposure to the progestin levonorgestrel on zebrafish (*Danio rerio*) fitness and breeding

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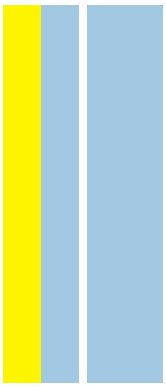

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Combined effects of increased temperature and exposure to the progestin levonorgestrel on the zebrafish (*Danio rerio*) fitness and breeding

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“The mind that opens to a new idea never returns to its original size”

Oliver Wendell Holmes

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Abstract

Climate change is unequivocal and consequently, many habitats and species can be altered or extinguished in a near future. According to the Intergovernmental Panel on Climate Change (IPCC) it is expected that global temperature will increase up to 4 ºC until the end of the 21st century which will be reflected also in the aquatic systems. Global warming is currently viewed as unavoidable, being the differences in temperature able to affect the fate, persistence, and bioaccumulation of some contaminants, such as endocrine disrupter compounds (EDCs). EDCs, such as the synthetic progestins, are frequently detected in surface and ground waters and they can interfere with the normal endocrine function of aquatic organisms, with all the potential to disturb reproduction.

To our knowledge, the research on the interactive effects of the combination of climate change and EDCs in aquatic organisms is quite scarce. Thus, the great novelty of this work is that it studies the combinatory effects of temperature and a synthetic progestin, levonorgestrel (LNG) on the fitness, reproduction and gonadal maturation kinetics of the adult zebrafish (*Danio rerio*) for 21 days. A factorial design manipulating temperature [setting as baseline present-day temperature of 27ºC, within the usual range of zebrafish husbandry, against warming (+3ºC)] and the progestin LNG [environmental (10 ngL^{-1}) and supra-environmental (1000 ngL^{-1}) concentrations] was considered.

The results of this study revealed no significant effects of temperature and LNG on the fitness condition of zebrafish, as well as on the gonadosomatic index. However, zebrafish fecundity was significantly negatively affected by the exposure to the LNG and the temperature seemed to have exacerbated the impacts of the progestin. In fact, the individuals exposed to the highest LNG concentration (at both temperatures) did not reproduce, but even those exposed to the lowest concentration but at a higher temperature also did not reproduce. These results can be explained, at least partially, based on the analysis of the ovary maturation stages. According to our histological findings, the higher the LNG concentration the lower the degree of maturation of the ovary. On the other hand, eggs eclosion rates were very dependent on the temperature, as expected. The higher the temperature the faster the eggs eclosion. In addition, exposure to the LNG, even at low concentrations, seemed to have negatively affected the eggs eclosion rates. Data on males are limited, but the testes of animals kept at the lowest temperature and highest LNG amount did not differ from controls in maturation status. This suggests that effects of the tested conditions may be more evident in females, which is worth exploring.

Considering the status of the zebrafish as a model organism, our findings suggest that in a future scenario of global warming and chemical exposure, the reproduction of at least some fish can be compromised which can put at risk the success of such species. Further research on this kind of topic is extremely important in order to better understand the interactive effects of the dual stresses on the functioning of aquatic ecosystems, and in this way contribute to a better management and conservation of those ecosystems.

Resumo

As alterações climáticas são inequívocas e, consequentemente, muitos habitats podem ser bastante alterados e espécies extintas num futuro próximo. De acordo com o Painel Intergovernamental para as Alterações Climáticas (IPCC) espera-se que a temperatura global aumente até 4 ºC até ao final do século 21, com o aumento a ser também refletido nos sistemas aquáticos. O aquecimento global é atualmente visto como inevitável, sendo as diferenças na temperatura capazes de afetar o destino, a persistência e a bioacumulação de certos contaminantes, tais como compostos disruptores endócrinos (EDCs). Os EDCs, tais como as progestinas sintéticas, são frequentemente detetados em águas superficiais e subterrâneas, podendo interferir com a homeostasia do sistema endócrino de espécies aquáticas, com potencial para perturbar a sua reprodução.

Tanto quanto sabemos, há muito poucos estudos sobre os efeitos interativos da combinação de alterações climáticas e de EDCs em organismos aquáticos. Assim, a grande inovação deste trabalho é o estudo dos efeitos combinatórios da temperatura e da exposição à hormona sintética levonorgestrel (LNG), durante 21 dias, na condição física, reprodução e na cinética da maturação da gónada do peixe-zebra adulto (*Danio rerio*). Foi feita uma experiência com dois fatores, em que foi considerada a temperatura [estabelecendo como referência base a temperatura ambiente de 27 ºC, dentro do limite habitual de criação da espécie, contrastando com outra a simular aquecimento (+3ºC)] e a hormona LNG [concentração ambiental (10 ngL-1) e supra-ambiental (1000 ngL-1)].

Os resultados deste estudo não revelaram efeitos significativos da temperatura e do LNG na condição física do peixe-zebra bem como no índice gonadossomático. Contudo, a fecundidade do peixe foi significativamente afetada de forma negativa pela exposição ao LNG e a temperatura parece exacerbar os efeitos da hormona. De facto, os animais expostos à concentração mais alta de LNG (nas duas temperaturas) não se reproduziram, e mesmo os expostos à concentração mais baixa de LNG, mas à temperatura mais alta, não se reproduziram. Estes resultados podem ser explicados, pelo menos parcialmente, com base na análise dos estádios de maturação do ovário. De acordo com os nossos resultados histológicos, quanto mais alta a concentração de LNG menos desenvolvidos foram os graus de maturação do ovário. Por outro lado, a taxa de eclosão dos ovos foi dependente da temperatura, tal como esperado. Quanto mais alta a temperatura, mais rápida foi a eclosão dos ovos. Além disso, a exposição ao LNG, mesmo na mais baixa concentração, parece afetar de forma negativa a taxa de eclosão dos ovos. Apesar dos dados em machos serem limitados, verificou-se que os testículos dos animais mantidos à temperatura mais baixa e à alta concentração de LNG não diferem dos estádios de maturação do controlo. Tal parece sugerir que os efeitos das concentrações testadas possam ser mais evidentes nas fêmeas; um aspeto que merece ser mais estudado.

Considerando o estatuto do peixe-zebra como organismo modelo, os nossos resultados sugerem que em cenários de aquecimento global e de exposição química a EDCs, a reprodução de (pelo menos) alguns peixes pode ser comprometida, podendo colocar em risco o sucesso reprodutivo de tais espécies. Concluindo, é de extrema importância pesquisar mais sobre este tópico, de forma a entender melhor os efeitos interativos de múltiplos estressores na funcionalidade dos ecossistemas aquáticos, e, desta forma, poder-se contribuir para uma melhor manutenção e conservação destes ecossistemas.

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Abbreviations

EDCs – Endocrine disrupter compounds

IPCC – Intergovernmental Panel on Climate Change

POPs – Persistent organic pollutants

PR – Progesterone receptor

ER – Estrogen receptor

AR – Androgen receptor

GR – Glucocorticoid receptor

MR – Mineralocorticoid receptor

P – Progestogenic

E – Estrogenic

AA – Anti-androgenic

A – Androgenic

ND – No data

P4 – Progesterone

MPA – Medroxylprogesterone acetate

MEP – Medroxylprogesterone

CMA – Chlormadinone acetate

CPA – Cyproterone acetate

NGA – Nomegestrol acetate

NES – Nestorone

TRI – Trimegestone

ETH – Ethisterone

NET – Noresthisterone

NEA – Noresthisterone diacetate

EDA – Ethynodiol diacetate

DIE – Dinogest

LNG – Levonorgestrel

ENG – Etonogestrel

NGMN – Norelgestromin

DSG – Desogestrel

NTE – Norgestimate

GES – Gestodene

DRO – Drospirenone

CAS – Chemical abstracts service

SD – Standart deviation

GSI – Gonadosomatic index

OECD – Organisation for Economic Co-operation and Development

SPE – Solid phase extraction

UPLH – Ultra-High Pressure Chromatography

PDA – Personal Digital Assistant

HPLC – High-performance liquid chromatography

LOD – Limits of detection

LOQ – Limits of quantification

K – Fulton’s condition index

W – Mean weight

TL – Mean total length

G – Gonad weight

W_B – Body weight

%V – Viability rate

%F – Fertility rate

%S – Survival rate

%E – Eclosion rate

Ct – Control

L1 – LNG (10 ngL^{-1})

L2 – LNG (1000 ngL^{-1})

Vv – Volume densities

EE_2 – 17α-ethinylestradiol

NP – Nonylphenol

OP – 4t-octylphenol

E_2 – 17β-estradiol

MDHT – 17-methyldihydrotestosterone

CNS – Central nervous system

Chapter I: Introduction

1. INTRODUCTION

1.1. Climate change

Climate change is unequivocal and as a consequence, a lot of habitats and species can be altered or extinguished in a near future (Maack and Segner, 2004; Manciocco et al., 2014).

According to the Intergovernmental Panel on Climate Change (IPCC) it is expected that global temperature will increase up to 4 ºC until the end of the 21st century which will be reflected also in the aquatic systems (IPCC, 2013). Global warming is unavoidable and the differences in temperature can affect the fate, persistence and bioaccumulation of some contaminants (Maack and Segner, 2004; Noyes and Lema, 2015). The temperature increase predicted by the IPCC can lead to changes in the structure, function and services of ecosystems and the worst scenario is if species cannot adapt to the new conditions, so it can lead to an huge extinction of several species (Schiedek et al., 2007; Manciocco et al., 2014; Noyes and Lema, 2015).

Temperature has large effects on the physico-chemical processes that dictate how biological systems function. Ectothermic organisms, those that cannot regulate body temperature using endogenous heat production, are particularly vulnerable to changes in environmental temperature (Schiedek et al., 2007; Scott and Johnston, 2012). The physiological and molecular mechanisms underlying the way ectotherms perform when faced with daily and seasonal temperature variation have attracted significant attention for decades, and this interest strengthened in an effort to understand the potential ecological impacts of global climate change. Higher temperatures rise the uptake rates of pollutants via changes in respiration rate in response to an increased metabolic rate and decrease in oxygen solubility (Schiedek et al., 2007; Scott and Johnston, 2012).

Other consequence of the increasing temperature is its potential to alter the environmental distribution and characteristics of chemical toxicants, suspected to be endocrine disruptors at environmentally relevant levels of human exposure (Maack and Segner, 2004; Manciocco et al., 2014).

Several researches are made about the effects of climate changes in various species, that the climate changes can affect the sensitivity and susceptibility of these species, but the efforts are being intensifying to know how multiple stressors contribute to various effects of climate changes via additive or synergistic interactions (Noyes and Lema, 2015; Allison et al., 2009). Climate changes has broad consequences in numerous chemical toxicants as referred before, including endocrine disrupter compounds (EDCs),

such as persistent organic pollutants (POPs) like dioxins and related compounds (Tanabe, 2002; Lamon et al., 2009; Noyes et al., 2009).

Valle et al. (2007) showed that climate changes have a huge influence on POPs environmental behaviour and fate, such as POPs mobility. Direct effects of climate changes such as wind patterns, sea ice, snow melting, precipitation patterns, alteration in salinity and changes in oceanic currents can alter the partitioning of POPs among the environmental compartments (Lamon et al., 2009; Noyes et al., 2009). Beyond that, some studies show that global warming has effects on the mode of action of EDCs, and increasing temperatures may potentiate the effect of such compounds (Prunier et al., 1997; Hooper et al., 2013).

Much is known about the impacts of climate change, mainly as single drivers, on the structure and functioning of aquatic ecosystems (Allison et al., 2009; Isaak et al., 2012). However, there are few studies regarding the combined effects of climate change and contaminants, like the molecules of the heterogeneous group of the endocrine disrupter compounds (EDCs) (Schiedek et al., 2007; Scott and Johnston, 2012; Moe et al., 2013).

1.2. Endocrine disrupter compounds

Over the last decades, a large amount of pharmaceuticals has been detected in the environment, because its consumption has been drastically increasing (Noyes et al., 2009; Christen et al., 2010). Natural and synthetic steroid hormones are known as one of the most active pharmaceutical classes. These compounds are frequently detected in surface and ground waters and they can interfere with the normal endocrine functions of aquatic organisms (Tanabe, 2002; Xu et al., 2008; Noyes et al., 2009).

Progestins are a synthetic version of the female reproductive hormone, progesterone (Hill and Janz, 2003; Scott and Johnston, 2012). Synthetic progestins, also known as gestagens or progestogens, mimic the effects C-21 steroid hormone, the natural hormone progesterone, involved in regulation of the menstrual cycle, pregnancy and embryogenesis in humans and in other species that is produced and secreted normally in female by the corpus luteum, the placenta and in small quantities by the adrenal cortex (Schindler et al., 2003; Besse and Garric, 2009). Accordingly, synthetic progestins are much used in human and veterinary medicine (Hill and Janz, 2003). They can be used in oral contraceptives either alone or in combination with estrogens (De Melo, 2010). The uses also include contraceptive treatments for correction of abnormal uterine bleeding,

controlling the symptoms of menopause and also for preventing certain types of cancer (Schindler et al., 2003; Besse and Garric, 2009).

To offer an idea of the huge amount of usage of progestins in human health, it is estimated that more than 100 million of women worldwide use hormonal contraceptives which act through progestogenic modes of action. In addition, the consumption of hormonal contraceptives tends to grow in the near future, which can lead to an increase of the progestins levels in aquatic systems in the next decades (Hoffmann and Kloas, 2012).

Synthetic progestins are very frequently detected in surface and ground waters, particularly water bodies that receive wastewater treatment plant effluents (Tanabe, 2002; Hill and Janz, 2003; Noyes et al., 2009). This indicates that such compounds are not completely removed by wastewater treatment processes. Additionally, this type of compound can be also released into aquatic environment from pharmaceutical industries and agricultural areas (e.g. farm animal waste). Based on the consumption increasing trend for the next decades, it is expected that concentrations found in water bodies will tend to increase proportionally (Tanabe, 2002; Hill and Janz, 2003; Noyes et al., 2009).

Forty years ago, some of the first progestins were introduced for clinical use but not without some negative side effects in humans, like androgenic activity (Hannah et al., 2002; Spitsbergen and Kent, 2003). Since then, countless synthetic progestins have been commercialized worldwide (Van den Belt et al., 2003; Manciocco et al., 2014). Actually, the news generations of progestins have fewer negative impacts, because they are more specific for the progesterone receptor (PR) (Kime and Nash, 1999).

Synthetic progestins, as well as the natural progesterone, act through nuclear receptors, mainly the progesterone receptor (PR), but also through other receptors such as the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). In accordance, synthetic progestins can have different hormonal activities and these are estrogenic, anti-androgenic and androgenic (Besse and Garric, 2009; Contardo-Jara et al., 2011).

Progestins are classified relatively to their chemical structure and can be subdivided in four groups that present similarity either to progesterone or testosterone. Progestins related to progesterone are subdivided into pregnanes and norpregnanes, while, those related to testosterone can be subdivided into those with and without a 17-ethinyl group, estrane and gonade group, respectively. The differences between pregnanes and norpregnanes group is the presence of methyl group in C10 position for pregnanes and the absence of this group in that position in norpregnanes group. The estrane and gonane group have a methyl group at the C13 position. There are huge

differences in the biological effects of progestins, with some presenting estrogenic activity while others have anti-androgenic action, such different properties are summarized in Table 1 (Stanczyk, 2003; Van den Belt et al., 2003; Besse and Garric, 2009).

Table 1 - Classification of Progestins (P – Progestogenic; E – Estrogenic; AA – Anti-androgenic; A – Androgenic and ND – No data) (adapted from Besse and Garric, 2009 and Kumar et al., 2015).

Classification	**Structural derivation**	**Progestin**	**Molecular formula**	**Properties**
Natural		Progesterone (P4)	$C_{21}H_{30}O_2$	P
Structurally related to progesterone	17-α-Hydroxyprogesterone	Medroxyprogesterone acetate (MPA)	$C_{24}H_{34}O_4$	P
		Medroxyprogesterone (MEP)	$C_{22}H_{32}O_3$	ND
		Chlormadinone acetate (CMA)	$C_{23}H_{31}ClO_4$	P / AA
		Cyproterone acetate (CPA)	$C_{24}H_{29}ClO_4$	P / AA
	19-Norprogesterone	Nomegestrol acetate (NGA)	$C_{23}H_{30}O_4$	P
		Nestorone (NES)	$C_{23}H_{30}O_4$	ND
		Trimegestone (TRI)	$C_{22}H_{30}O_3$	ND
Structurally related to testosterone	19-Nortestosterone (Estranes)	Ethisterone (ETH)	$C_{21}H_{28}O_2$	ND
		Noresthisterone (NET)	$C_{20}H_{26}O_2$	P / E / A
		Noresthisterone acetate (NEA)	$C_{22}H_{28}O_3$	ND
		Ethynodiol diacetate (EDA)	$C_{24}H_{32}O_4$	ND
		Dienogest (DIE)	$C_{20}H_{25}NO_2$	P / AA / A

	19-Nortestosterone (Gonanes)	Levonorgestrel (LNG)	$C_{21}H_{28}O_2$	P / A
		Etonogestrel (ENG)	$C_{22}H_{28}O_2$	P / A
		Norelgestromin (NGMN)	$C_{21}H_{29}NO_2$	ND
		Desogestrel (DSG)	$C_{22}H_{30}O$	P / A
		Norgestimate (NTE)	$C_{23}H_{31}NO_3$	P / A
		Gestodene (GES)	$C_{21}H_{26}O_2$	P / A
	Spirolactone derivative	Drospirenone (DRO)	$C_{24}H_{30}O_3$	P / AA

1.2.1. Levonorgestrel

Levonorgestrel (LNG), a synthetic progestin, is the most commonly used in contraceptive pills, contraceptive implants and emergency contraceptive pills, since its development in 1972 (Contardo-Jara et al., 2011; Hua et al., 2015; Runnalls et al., 2015; Frankel et al., 2016). LNG has been detected, worldwide, in freshwater systems and sewage treatment plant effluents, at low concentrations in the range of ngL^{-1} (Hua et al., 2015; Runnalls et al., 2015). However, LNG represents a potential risk to aquatic organisms due to the particles that stay in several concentrations in aquatic environment. In addition, LNG has a high capacity of bioaccumulation in fish (Hua et al., 2015) and it has a strong androgenic activity and activate fish androgen receptors as well as triggers the development of male secondary characteristics in females (Hua et al., 2015; Liang et al., 2015; Runnalls et al., 2015; Frankel et al., 2016).

Although many studies have been published on the progestins' effects, namely about LNG, (Hua et al., 2015; Runnalls et al., 2015; Frankel et al., 2016), a lack of information regarding combined effects between environmental factors and endocrine disrupters is found in the literature. The great novelty of this study is that it evaluates the combined effects of both stressors (temperature and LNG) on the well-studied zebrafish (*Danio rerio*).

1.3. Zebrafish

Zebrafish (*Danio rerio*) originally from Southeast Asia is a freshwater species from tropical climates and is considered an excellent model for animal experimentation, since it offers practical and technical advantages to study biological processes, effects and mechanisms and is representative of a large group of organisms. The technical advantages are the low cost and easy maintenance, the high fecundity and, the easy manipulation, among others (Spitsbergen and Kent, 2003; Hill et al., 2005; Segner, 2009; de Esch et al., 2012).

Zebrafish is frequently used as a model in ecotoxicological studies, in order to investigate the chemical effects of several compounds at different functional aspects, such as sex differentiation, behavioural aspects, among others (Silva et al., 2012; Santos et al., 2014; Hua et al., 2015).

This species has the particularity to have a quite fast embryonic development which makes it to be highly used for reproduction studies (Kimmel et al., 1995; D'Costa and Shepherd, 2009). Other reason for the generalized use of zebrafish embryos is the external development of the fertilized eggs which makes possible to follow in detail all embryonic development stages and this is relevant for developmental biology and genetics studies (D'Costa and Shepherd, 2009).

1.4. Objectives

Attending to the lack of information in the literature regarding the interaction of climate factors and endocrine disruptors (namely progestins), the main goal of this work was to evaluate the combined effects of temperature (in view of global warming) and the synthetic progestin, LNG on the fitness, reproduction and gonadal maturation kinetics of the adult zebrafish. For that, several endpoints were evaluated, such as:

- Fish fitness condition (pre-reproduction);
- Gonadosomatic index (pre- and post-reproduction);
- Reproduction parameters (fecundity rate, survival rate and viability rate);
- Gonadal maturation kinetics (pre- and post- reproduction).

Chapter II: Materials and Methods

2. MATERIALS AND METHODS

2.1. Chemical

Levonorgestrel (LNG; CAS 797-63-7; purity = 99 %) was purchased from Sigma-Aldrich (St. Louis, USA).

All stock solutions were prepared with analytical ethanol supplied by Merck (Darmstadt, Germany) and stored in the dark at -20 ºC.

2.2. Test animals

Adult male and female of zebrafish (*Danio rerio*) with the mean body weight of 0.58 ± 0.15 g (mean ± SD) and body length of 3.94 ± 0.26 cm were obtained from local suppliers. During the acclimation period, they were kept in a 150 L aquarium with dechlorinated and aerated water for 30 days with water filtration. The temperature was maintained at 27 ± 1 °C and the photoperiod at 14:10 (light:dark cycle). During this period, zebrafish were fed with commercially available compound feed, Tetramin (Tetra, Germany) with a minimum of 47% of protein and 10 % of lipids, once a day before the water renewal.

2.3. Experimental design

After the initial acclimation period, a factorial design manipulating temperature [present-day temperature and warming (+ 3 ºC)] and the progestin LNG (environmental and supra-environmental concentration) was considered. Zebrafish were exposed to six treatments: T_1) 27 ºC – the ambient temperature and solvent control (0.01 % ethanol); T_2) 27 ºC and LNG (10 ngL^{-1}, corresponding to the environmental concentration); T_3) 27 ºC and LNG (1000 ngL^{-1}, corresponding to a supra-environmental concentration); T_4) 30 ºC – the future warming scenario for freshwater aquatic systems (+ 3 ºC) and solvent control (0.01 % ethanol); T_5) 30 ºC and LNG (10 ngL^{-1}); T_6) 30 ºC and LNG (1000 ngL^{-1}).

Each treatment included 6 replicates (glass flasks of 4.5 L each) that were distributed randomly by 6 water baths in order to maintain the temperature constant (see Figure 1). Five zebrafish (female:male = 3:2) individuals were added to each of the 36 glass flask replicates.

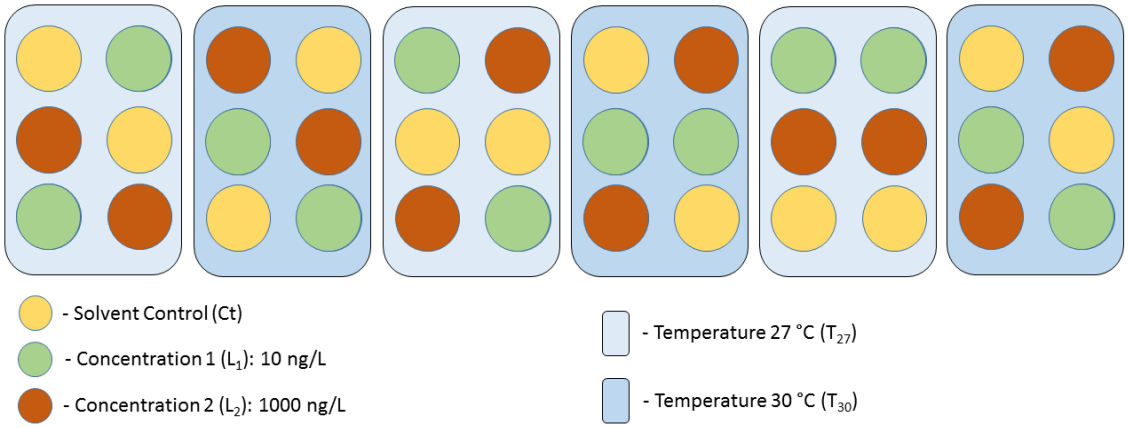

Figure 1 - Illustration of the experimental set-up during the 21 days' exposure period.

The experiment was divided in two phases: 1) pre-reproduction and 2) post-reproduction. In the first one, zebrafish were exposed to the different treatments for 21 days. In the second phase, the exposure to the hormone LNG was stopped and individuals were allowed to reproduce.

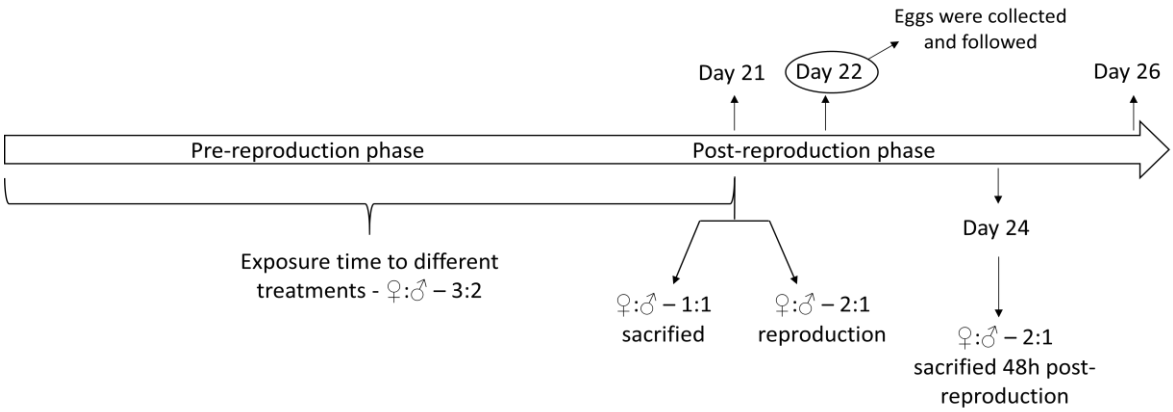

Figure 2 - Timeline of the work stages: Pre-reproduction phase and Post-reproduction phase.

During the experiment, the exposure conditions were maintained as follows: water temperature, 27 ± 1 °C and 30 ± 1 °C; light regime, 14 h light and 10 h dark; pH, 8.27 ± 0.20; dissolved oxygen, 96.42 ± 4.97 %. Exposure media were renewed daily (100 %) to ensure the quality of the water and guarantee the LNG concentration; renewals were done quite gently to minimize fish stress. The water physicochemical parameters such as pH, dissolved oxygen, nitrites and ammonia were measured three times a week, except for temperature that was measured daily. The mean values are shown in Table 2. During the exposure period, zebrafish were fed once a day, alternatively with Tetramin and with hatched brine shrimp *Artemia salina* (Ocean Nutrition, Belgium).

Table 2 - Physiochemical parameters of water (mean ± SD) at two temperatures, 27º and 30 ºC, during the experiment.

	pH	Dissolved oxygen (%)	Ammonia (mgL^{-1})	Nitrite (mgL^{-1})
27 ºC	8.26 ± 0.17	97.11 ± 4.88	0.13 ± 0.13	0.20 ± 0.25
30 ºC	8.29 ± 0.22	95.73 ± 5.06	0.10 ± 0.04	0.12 ± 0.18

After 21 days' exposure period, one male and one female fish from each replicate were removed and sacrified with a letal concentration of 2-phenoxyethanol at 0.1% (v/v) dissolved in water (Sigma, Switzerland), by prolonged immersion, until total cessation of opercular movement. Then, the fish were patted dry with paper towel and total body weight and length of each fish were recorded. Afterwards, the gonads were collected at necropsy, immediately putted in a fixative, Greenfix (Diapath, Italy) for 48 h and then processed for histology.

The remaining fishes in each of the glass flasks were transferred to cages for reproduction. Each cage bottom was covered with glass marbles and immersed in a 5 L aquarium subject to the same temperature and photoperiod conditions as in the pre-reproduction phase. Male and female (1:2) were first kept each in its compartment, but the separator was removed before turning off the lights in the evening. Fish were thus kept together during the night for mating and breeding. In the following day, fish were removed from 60 to 90 min after the light onset and the cages were checked for the presence of eggs. These were collected and rinsed several times with autoclaved deionized water to remove debris. The fertilized eggs were maintained in the same conditions and were cleaned once a day; the water parameters (pH, dissolved oxygen, nitrites, ammonia and temperature) were measured in the second and third day after posture. Eggs were checked daily during 5 days (96 h) for mortality and unhatching.

After reproduction, the fishes were also euthanized with 2-phenoxyethanol 0.1 % (v/v). The weight and length of the body were measured, and the gonad was excised at necropsy, weighted and immediately fixed in as above and then processed, according with the procedures detailed below.

2.4. Sampling and histological preparation

After euthanasia by a lethal dose of anaesthetic, a ventral-lateral incision uncovered the body cavity. Then, the gonads were carefully excised and weighed (mg). The result was expressed as a function of the total body weight in order to obtain the gonadosomatic index (GSI), for the pre- and post-reproduction phases. As mencioned, the gonads were fixed in Greenfix for 48 hours at room temperature. This fixative is a formaldehyde-free mixture formulated by Diapath (Italy), with no-carcinogenicity and other toxicant effects attributed to that aldehyde, while having in general similar efficiency as the common formalin (Gatta et al., 2012). Each gonad was divided in half and stored in 70 % ethanol. Each piece was processed for two embedding media: paraffin (Histosec, Merck, Germany) and methacrylate (Technovit 7100, Heraeus Kulzer, Germany).

2.4.1. Paraffin

After fixations and storage in 70 % ethanol, the fragments were processed in an automatic tissue processor (Leica TP1020, Germany) according to the protocol described in Appendix I. The embedding in paraffin wax (Histosec, Merck, Germany) at 60 ºC was performed in the Leica EG 1140H Embedding Center. Sections (4 µm in thickness) were obtained with disposable knifes using a fully motorized rotary microtome (Leica RM2155, Germany). The slides were stained with haematoxylin-eosin (Appendix II).

2.4.2. Methacrylate

Also following storage in 70 % ethanol, the pieces were manually dehydrated in increasing concentration of ethanol (90 %, 96 % and 100 %). Gonads were embedded in methacrylate resin Technovit 7100 (Heraeus Kulzer, Germany) using an appropriate plastic mold, following the steps specified by the manufacter and included in the Technovit product information as described in Appendix III. Gonads were embedded in random orientation, and blocks were hardened overnight. Blocks were sectioned on fully motorized rotary microtome (Leica RM2155, Germany), at a nominal thickness of 4 µm, using a tungsten carbide knife. Sections were floated in a water bath (25 ºC) for stretching, picked on StarFrost slides and then placed in a hot plate (60 ºC) for 1 hour (Appendix III). The slides were stained with an adapted protocol of haematoxylin-eosin method (Appendix IV).

2.5. Histological semi-quantitative and qualitative analyses

2.5.1. Grading of gonadal maturation

For the semi-quantitative grading of gonads, representative sections of fish from all treatments were examined under a brightfield microscope (Olympus BX 50, Japan), with the analyses being made under up to the ×40 objective lens, for males and females. To classify the samples according to the maturation stage of the gonad it was followed the OEDC protocol, in which were adopted five stages for testis and six stages for ovaries (Johnson et al., 2009). Our study was initially made using paraffin sections. Later, data were further confirmed in plastic sections when making the stereological study (see below); to this point of the study, only the females were analysed by this refined strategy.

For testis the five stages analysed were the following: a) stage 0 (undeveloped) the gonad presents entirely immature phases (spermatogonia to spermatids) with no spermatozoa; b) stage 1 (early spermatogenic) the immature phases predominate, but spermatozoa may also be observed and the germinal epithelium is thinner than it is during stage 2; c) stage 2 (mid-spermatogenic) spermatocytes, spermatids and spermatozoa are present in roughly equal proportions and the germinal epithelium is thinner than stage 1, but thicker than stage 3; d) stage 3 (late spermatogenic) all stages may be observed, however, mature sperm predominate and the germinal epithelium is thinner than it is during stage 2; and finally e) stage 4 (spent) it can be found loose connective tissue with some remnant sperm.

For ovaries, six stages were considered: a) stage 0 (undeveloped) the gonad consists entirely in immature phases (oogonia to perinucleolar oocytes), without cortical alveoli; b) stage 1 (early development) the vast majority are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar; c) stage 2 (mid-development) at least half of observed follicles are early and mid-vitellogenic; d) stage 3 (late development) the majority of developing follicles are late vitellogenic; e) stage 4 (late development/hydrated) the majority of follicles are late vitellogenic and mature/ spawning follicles, the follicles are larger as compared to stage 3; and finally f) stage 5 (post-ovulatory) the gonad consists predominantly in spent follicles, remnants of theca externa and granulosa.

2.5.2. Stereological analyses in methacrylate sections

In order to quantitate the maturation of the ovary, its structural compartments were divided into the following 5 identifiable gamete stages: primary oocytes, cortical-alveolar oocytes, early vitellogenic oocytes, late/mature oocytes and atretic oocytes, plus the

connective tissue was also observed. Here, the term “oocyte” operationally means “oocyte-follicle complexes”. An additional structural compartment was the ovarian connective tissue (including the “nests” of proliferating oogonia). The target parameters were the relative volumes of the cited compartments in relation to the whole ovary. In order to quantify them each glass slide was scanned with a virtual slide scanner (Olympus VS110, Japan). The stereological procedures were applied to the resulting virtual slides. These were studied on a computer monitor and systematically sampled with the OlyVIA 2.8 Viewer (Olympus, Japan). The effort of sampling depended on the variable size of the gonads, and produced a minimum of 8 and a maximum of 35 fields. Every sampled field was subjected to a stereological probe that was superimposed over virtual images of the targeted tissues. The relative volumes were estimated using the principle of differential (manual) point counting as detailed in technical textbooks (viz., Weibel 1979). In summary, after pilot trials, a two-lattice (1:16) point grid was specifically designed with Microsoft’s PowerPoint. It was printed in a transparent sheet, which was superimposed to the computer monitor. The analysis was made digitally zooming 33x the original virtual slide magnification. At every sampled field the number of point falling over the ovary and over any of the structures of interest were counted. The lattice-grid used for every compartment depended of frequency, with the coarse 1:1 point ratio being used for the whole organ and common structures and the fine 1:16 point ratio targeted more rare compartments. Typically, oocyte-follicle complexes were probed with the finer lattice of 1:16 points, except for the late/mature oocytes that in gonads were they were frequent they were probed with the 1:1 lattice. The estimation of the relative volume (V_V) of a structure in relation to the reference space (ovary), for each animal, was estimated by the formula:

$$V_V = \frac{\sum Points\ over\ the\ structure\ of\ interest, over\ all\ fields}{Ratio\ of\ fine\ to\ coarse\ points \times \sum Points\ over\ the\ ovary\ , over\ all\ fields}$$

Due to time constrains males were not yet subjected to stereological analyses.

2.6. LNG analysis from water samples

2.6.1. Water sampling

Water samples (2 L, n = 3 replicate per treatment) from all exposure groups were collected in amber flasks just after the LNG addition (T_0) or before the water solution renewal (T_{24}). These samples were kept refrigerated (± 4 ºC), and immediately vacuum filtered through 0.45 µm glass fibre filters to remove suspended particles. Each filter was washed with approximately 2 mL of methanol (CH_3OH) and this volume was added to the

filtrate. All samples were adjusted to pH 2 with few drops of H_2SO_4 (conc.) and maintained at ± 4 ºC in dark until extraction. The last step occurred, always, within a maximum of 48 h after sampling (Appendix V).

2.6.2. Solid-phase extraction

LNG was extracted from water samples, by solid phase extraction (SPE) using OASIS HLB cartridges adapted in an off-line SPE vacuum extraction device (Waters). The breakthrough volume, pH adjustment, washes and elution conditions followed a method initially developed to extract phenolic compounds and steroids in water (Ribeiro et al., 2007). In this study, the last method was broadened for the analysis of LNG and is fully described in Appendix IV.

Briefly, the condition step was carried out with 13 mL of methanol:dicloromethane (CH_3OH:CH_2Cl_2, 50:50, v/v), 6 mL of CH_3OH and 13 mL of ultrapure Milli-Q water at a flow rate of 1 mL min^{-1}. Water samples were loaded onto SPE cartridges at a constant flow rate of 5 mL min^{-1} followed by a washing step with 13 mL of ultrapure Milli-Q water and 500 µL of methanol. Cartridges were dried under vacuum for 30 min and then mounted with 1 g Sep-Pak silica cartridges, previously conditioned with 10 mL of CH_3OH:CH_2Cl_2 (50:50, v/v), at 1 mL min^{-1}. The resulting extracts were evaporated to dryness in a heating block at 40 ºC under a gentle steam of nitrogen and reconstituted in 20 µL of acetonitrile (C_2H_3N) before analysis. This method successfully allowed the recovery of LNG (103.7 %) with a RSD of 6.6 %.

2.6.3. Quantification by Ultra-High Pressure Liquid Chromatography (UPLC)

The UPLC analysis was performed using a liquid chromatograph coupled with a high-speed PDA detector an autosampler and an oven all from Thermo Scientific analytical instruments (Accela™). The analytic column was the Acquity UPLC BEH C_{18} column (1.7 µm, 2.1x100 mm, no. 186002352) from Waters™. Acetonitrile HPLC grade (≥99.93%) and ultrapure Milli-Q water (50:50, v/v), previously filtered and degassed, was maintained isocratic at a constant flow rate of 350 µL min^{-1}. The oven temperature was programmed for 40 ºC. Finally, 5 µL was injected into the UPLC system for analysis.

LNG was isolated from matrix extremely fast, only 1.1 min, and its limits of detection (LOD) and quantification (LOQ) of this method were 1.2 ng L^{-1} and 4.0 ng L^{-1}, respectively.

2.7. Fulton's condition index (K)

Zebrafish length and wet weight data were used to determine the fitness condition of the organisms after 21 days' exposure. In this case, the Fulton's condition index (K) was applied and is, expressed by the following:

$$K = \frac{W}{TL^3} \times 100$$

Where: W = mean weight and TL = mean total length (Ricker, 1975).

2.8. Gonadosomatic index (GSI)

Zebrafish gonad and body weight data were used to determine the reproduction allocation and reproduction condition of organisms after 21 days after exposure during pre- and post-reproduction phases. In this case, it was used the gonadosomatic index (GSI), expressed by the following:

$$GSI = \frac{G}{W_B}$$

Where: G = gonad weight and W_B = body weight.

2.9. Reproductive parameters

2.9.1. Fecundity

Zebrafish fecundity can be defined by the total number of eggs released by females in each treatment tank during the reproduction period (Xu et al., 2008).

2.9.2. Viability, fertility, survival and eclosion rate

During the post-reproduction phase, eggs were collected and counted at 0 h, 8 h, 24 h, 48 h, 72 h and 96 h to evaluate different reproductive parameters, such as: viability rate (%V), fertility rate (%F), survival rate (%S) and eclosion rate (%E), expressed by the following:

$$\%V = \frac{viable\ eggs\ (8h)}{total\ number\ of\ eggs\ (0h)} \times 100$$

$$\%F = \frac{viable\ eggs\ (24h)}{total\ number\ of\ eggs\ (0h)} \times 100$$

$$\%S = \frac{viable\ eggs\ (96h)}{total\ number\ of\ eggs\ (0h)} \times 100$$

$$\%E = \frac{hatched\ eggs\ (48h/72h/96h)}{viable\ eggs\ (48h/72h/96h)} \times 100$$

Water was renewed and dead embryos were removed on a daily basis. The embryos were followed up during those 5 days (96 h) to evaluate hatching.

2.10. Data analysis

All data were previously examined for normality and homogeneity of variance using Kolmogorov-Smirnov's and Levene's tests, respectively. Data were transformed (arcsine, $\log_{10} + 1$) to achieve normality and homoscedasticity whenever necessary (Zar, 1999). Data that still did not conform with those assumptions were ranked-transformed following the same approach as Ko et al. (2014).

For Fulton's (K) and GSI (pre-reproduction), a 2-way ANOVA (temperature x LNG) was carried out to determine the differences among treatments, for males and females, separately.

Regarding GSI (post-reproduction), 1-way ANOVA was carried out, since due to the unbalanced design (absence of data on treatments L1T30, L2T27 and L2T30 due to non-reproduction) it was not possible to apply a 2-way ANOVA.

One-way ANOVAs were also carried out to test for differences in the reproductive parameters (viability, fertility and survival rates) among the different treatments (same reason as for the GSI post-reproduction).

A 2-way ANOVA on ranks (treatments x time) was used to test for differences in eclosion rates among treatments.

For fecundity, a 2-way ANOVA on ranks (temperature x LNG) was applied to test for differences among treatments. In addition, a multiple regression analysis was applied to test for relationships between temperature, LNG and the fecundity levels.

Regarding grading analysis (paraffin), it was carried out a 2-way ANOVA on ranks (for the pre-reproduction phase), for both male and female, to test for differences in the development stages of the gonads among treatments. For the post-reproduction phase, 1-way ANOVA on ranks was applied due to the unbalanced design.

For the stereological analysis (methacrylate resin), a 2-way ANOVA on ranks in pre-reproduction and a 1-way ANOVA on ranks in post-reproduction were used to test for differences on the volumes of the distinct structural compartments among treatments.

The statistical analysis was performed with STATISTICA software (version 13, StatSoft Inc.).

Chapter III: Results

3. RESULTS

3.1. Levonorgestrel concentrations in water

As expected, the LNG concentration in water from the solvent control (Ct) for both temperatures was lower than the detection limit (< 1.2 ngL^{-1}). In contrast, for both LNG concentrations (L1 and L2) the values were quite constant during the period between medium renewal, with no relevant decay all over the time.

Table 3 - LNG concentration (ngL^{-1}) in water during the experiment. The water was sampled just after renewal (T_0) and before renewal (T_{24}). Values represent mean ± SE.

Treatments		Nominal	T_0	T_{24}
27 ºC	Ct	-	< 1.2	< 1.2
	L1	10	11.1 ± 0.79	9.48 ± 0.04
	L2	1000	978.7 ± 17.81	917 ± 49.18
30 ºC	Ct	-	< 1.2	< 1.2
	L1	10	11.1 ± 0.79	8.49 ± 0.47
	L2	1000	978.7 ± 17.81	867.3 ± 24.2

3.2. Fulton's condition index (K)

Regarding the K, female zebrafish presented, in general, higher mean values than males; despite differences were not always significant. For the females, no significant differences were observed between treatments (2-way ANOVA, $p > 0.05$). A similar result was observed in the males; despite in L1T27 the K was lower than in the remaining treatments (Figure 2).

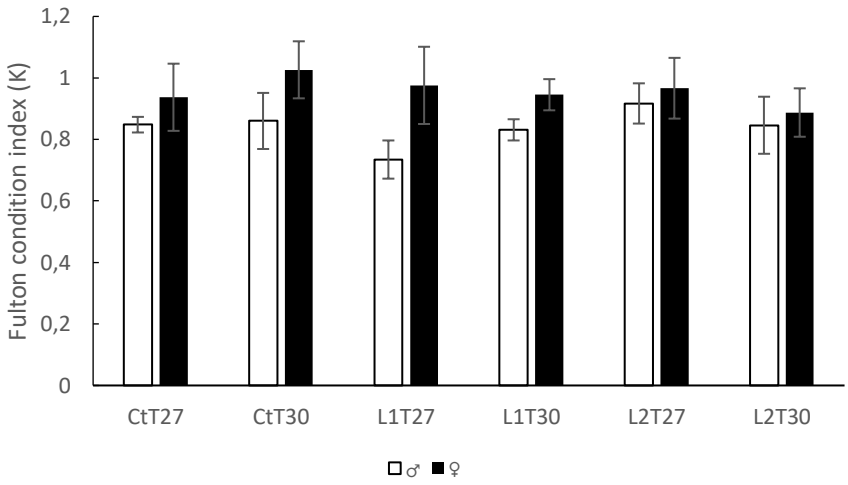

Figure 3 – Fulton's condition index (K) for the zebrafish exposed to different combinations of temperature and LNG concentrations. Values represent mean ± SD. CtT27 – solvent control (0.01 % ethanol) at 27 ºC, CtT30 – solvent control (0.01 % ethanol) at 30 ºC, L1T27 – LNG (10 ngL^{-1}) at 27 ºC, L1T30 – LNG (10 ngL^{-1}) at 30 ºC, L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

3.3. Gonadosomatic index (GSI)

3.3.1. Pre- and post-reproduction

In the pre-reproduction phase (Figure 3a), the females always presented GSI values well above the males. For the females there seems to be a slight decline trend on GSI values for all the treatments compared to the solvent control at ambient temperature, however, no significant differences were observed (2-way ANOVA, $p > 0.05$). Likewise, for the males, no significant differences between treatments were observed (2-way ANOVA, $p > 0.05$).

In the post-reproduction phase, only data for the solvent control and L1T27 are represented in Figure 3b, since individuals exposed to higher LNG concentrations and higher temperatures did not reproduce. Once again, the females presented higher values than the males. However, no significant differences between treatments were found for both females and males (1-way ANOVA and 1-way ANOVA on ranks, respectively, $p > 0.05$).

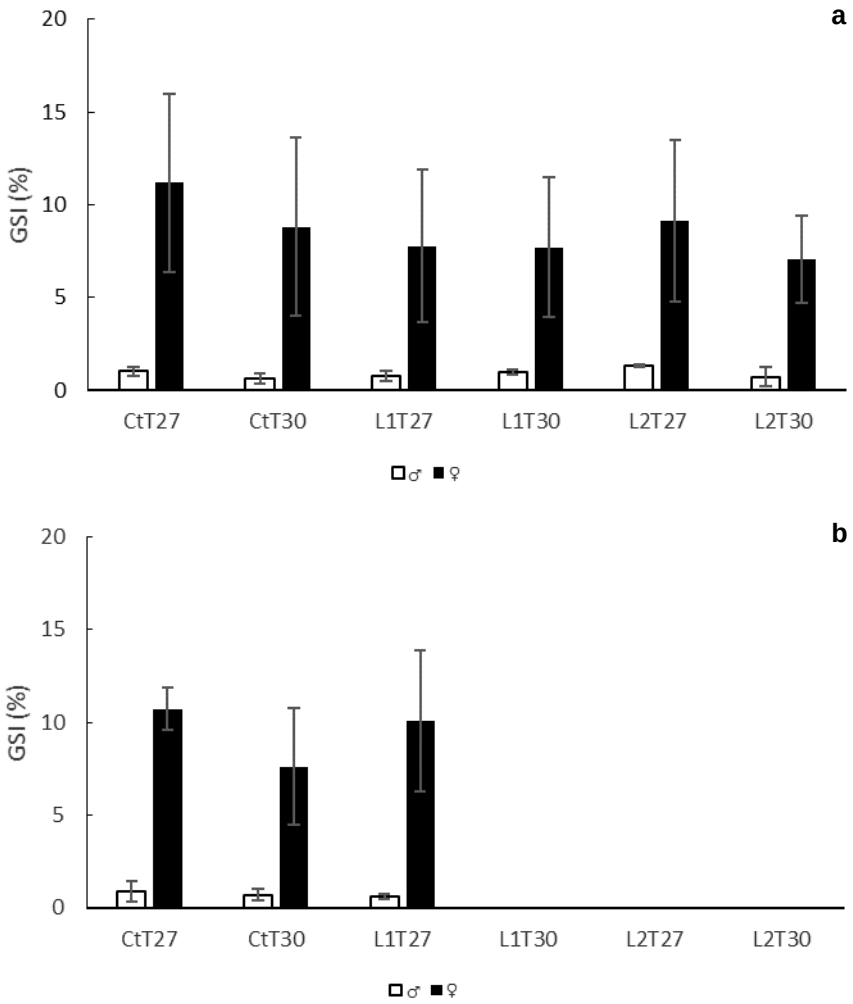

Figure 4 - Gonadosomatic index (GSI) for the adult zebrafish in two different phases: **a)** Pre-reproduction; **b)** Post-reproduction. Values represent mean ± SD. CtT27 – solvent control (0.01 % ethanol) at 27 ºC, CtT30 – solvent control (0.01 % ethanol) at 30 ºC, L1T27 – LNG (10 ngL^{-1}) at 27 ºC, L1T30 – LNG (10 ngL^{-1}) at 30 ºC, L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

3.4. Reproduction parameters

3.4.1. Fecundity

Regarding fecundity, it is possible to observe that the females from L1T30, L2T27 and L2T30 treatments did not reproduce at all (Figure 4). In addition, a decrease in fecundity with the increase of the LNG concentration and the temperature was clearly visible. Statistically, significant differences were found between LNG concentrations and the solvent control (2-way ANOVA on ranks, $F_{(2, 12)} = 137.348$, $p = 0$), as well as between both temperatures (2-way ANOVA on ranks, $F_{(1, 12)} = 23$, $p = 0.0004$). Also, a significant

interaction between LNG concentration and temperature (2-way ANOVA on ranks, $F_{(2, 12)} = 11.261$, $p = 0.002$) was detected.

Negative correlations were observed (multiple regression, $R^2 = 0.848$, $p < 0.000$) between, on the one hand, LNG concentrations and fecundity ($\beta = - 0.883$, $p = 0$), and, on the other hand, between temperature and fecundity ($\beta = - 0.263$, $p = 0.019$). Despite both factors were significant, the progestin had a higher negative effect on zebrafish fecundity than temperature.

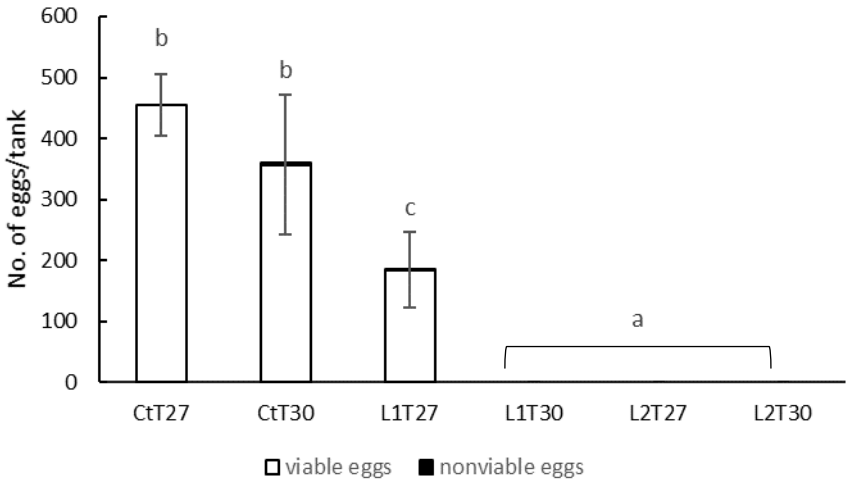

Figure 5 - Results of breeding trial of zebrafish after 21 days of LNG exposure. Zebrafish exposed to 10 ngL^{-1} at 30 ºC and 1000 ngL^{-1} at 27 ºC and 30 ºC exhibited complete reproductive failure with no eggs. Total bar length indicates the total number of eggs per treatment (mean ± SD). The lighter bar indicates the total number of viable eggs at 0 h per treatment. The black bar indicates the total number of nonviable eggs at 0 h per treatment. Different letters mean significant differences among treatments. CtT27 – solvent control (0.01 % ethanol) at 27 ºC, CtT30 – solvent control (0.01 % ethanol) at 30 ºC, L1T27 – LNG (10 ngL^{-1}) at 27 ºC, L1T30 – LNG (10 ngL^{-1}) at 30 ºC, L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

3.4.2. Viability, fertility and survival rate

Concerning reproduction parameters, for L1T30, L2T27 and L2T30 treatments, no data was obtained since the individuals exposed to those treatments did not reproduce under those conditions. For the viability rate (Figure 5a), the three treatments were very similar and no significant differences were observed among them (1-way ANOVA, $p > 0.05$). Likewise, for the fertility rate, no significant differences were found among treatments (1-way ANOVA, $p > 0.05$). Finally, for the survival rate, and despite the slight decline that seems to occur for the treatments CtT30 and L1T27 when compared to the

CtT27, no significant differences were found among treatments (1-way ANOVA, $p > 0.05$) (Figure 5).

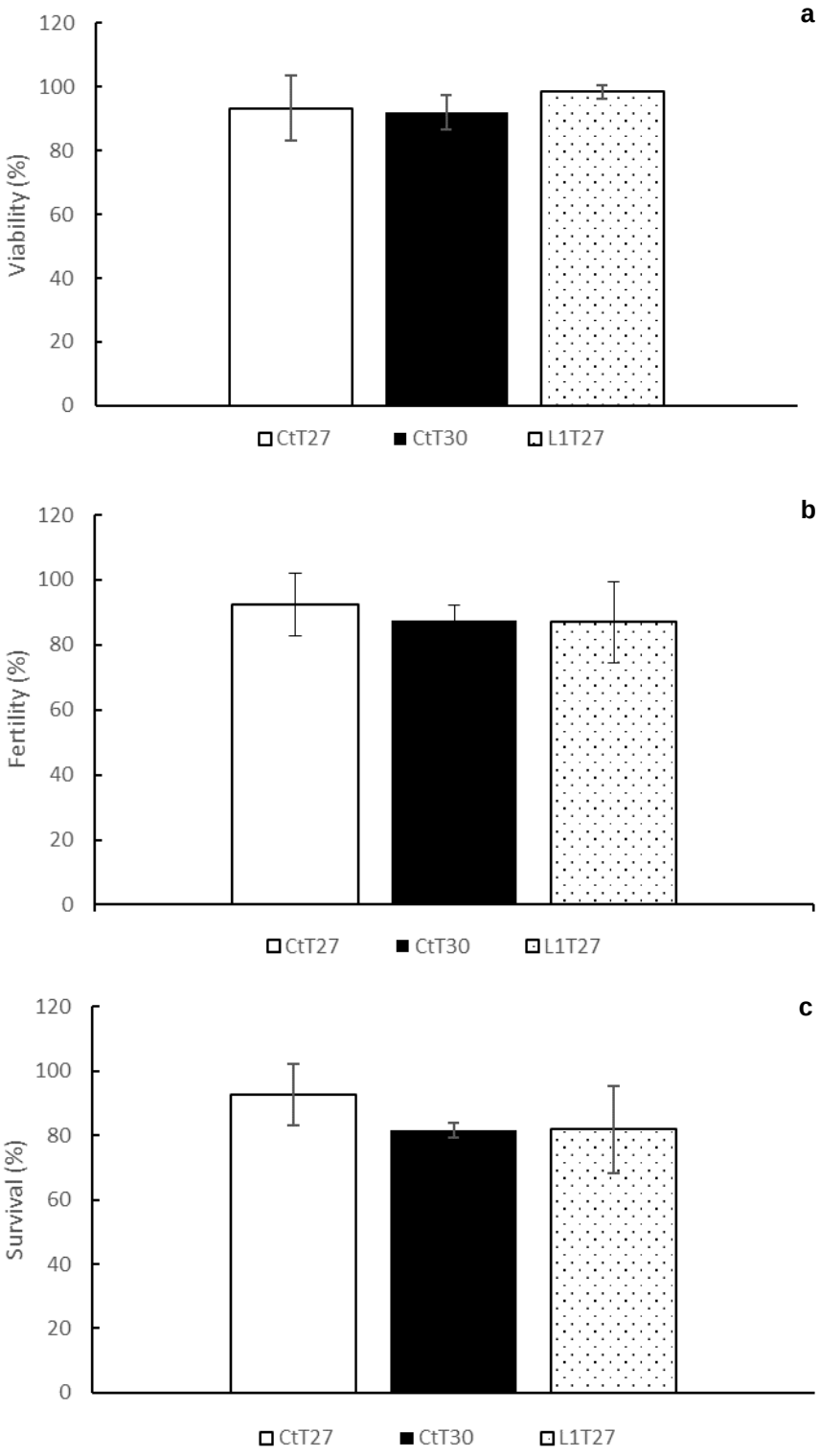

Figure 6 - Reproduction parameters for the zebrafish: **a)** Viability rate; **b)** Fertility rate and **c)** Survival rate. Values represent mean ± SD. CtT27 – solvent control (0.01 % ethanol) at 27 ºC, CtT30 – solvent control (0.01 % ethanol) at 30 ºC, L1T27 – LNG (10 ngL^{-1}) at 27 ºC.

3.4.3. Eclosion rate

The eclosion rate seem to be entirely related with temperature, since eggs exposed to warming conditions (30 ºC) hatched faster than those exposed to ambient temperature (27 ºC) (Figure 6). Almost 60 % of the eggs exposed to CtT30 hatched after 48 h, reaching approximately 90 % of eclosion after 72 h. On the other hand, treatments exposed to ambient temperature presented a vestigial eclosion rate after 48 h and just at 72 h almost 80 % of the eggs hatched. In addition, the treatment exposed to low LNG concentrations (10 ngL^{-1}) presented slightly lower eclosion rates than the controls. Significant differences were observed among treatments (2-way ANOVA on ranks, $F_{(2,18)} = 7.69$, $p = 0.004$) and eclosion times ($F_{(2,18)} = 23.40$, $p < 0.0001$).

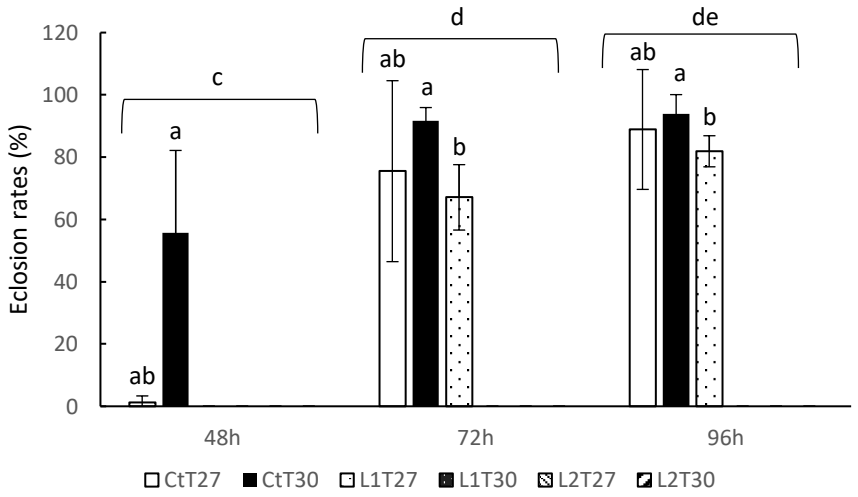

Figure 7 - Eclosion rate of the zebrafish eggs exposed to differents treatments after 48 h, 72 h and 96 h. Values represent mean ± SD. Different letters mean significant differences among treatments. Treatments with not bars (L1T30, L2T27 and L2T30) signify that the eclosion rate was zero. CtT27 – solvent control (0.01 % ethanol) at 27 ºC, CtT30 – solvent control (0.01 % ethanol) at 30 ºC, L1T27 – LNG (10 ngL^{-1}) at 27 ºC, L1T30 – LNG (10 ngL^{1}) at 30 ºC, L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

3.5. Grading analysis

3.5.1. Pre-reproduction

Qualitatively, histopathological aspects were seen neither in ovaries nor in testes. Regarding females, we found ovaries varying from stages 1 to 4 (Figure 7), but any in stages 0 (undeveloped) or 5 (post-ovulatory).

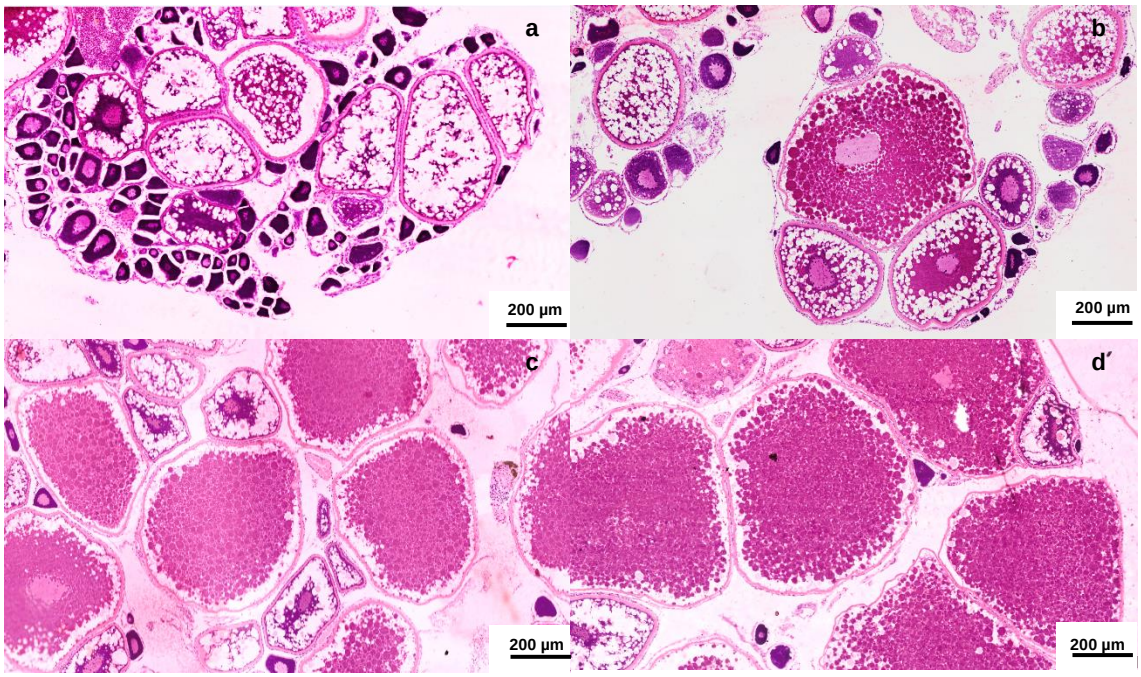

Figure 8 - Representative general histological aspects of the four stages of female grading. **a)** Stage 1 (early development) from L1T30 – LNG (10 ngL^{-1}) at 30 ºC; **b**) Stage 2 (mid-development) from L2T30 – LNG (1000 ngL^{-1}) at 30 ºC; **c)** Stage 3 (late development) from L1T27 – LNG (10 ngL^{-1}) at 27 ºC; **d**) stage 4 (late development/hydrated) from L2T30 - LNG (1000 ngL^{-1}) at 30 ºC.

When grouping females by their gonadal maturity, the grading analysis allowed us to observe a decline in the percentage of females with more matured gonads with the increase of LNG concentration, for both temperature scenarios (Figure 8), however, the increase of temperature exacerbated the effects of the hormone. A potentiation effect between temperature and LNG seems to be clear for the zebrafish. Analyses of the semi-quantitative ovary maturity grades among treatments revealed significant differences for the LNG concentration (2-way ANOVA on ranks, $F_{(2, 39)} = 7.0636$, $p = 0.0024$), however, for the temperature no significant differences were found (2-way ANOVA on ranks, $p > 0.05$). Accordingly, the ovary maturation was significantly more affected statistically by the LNG concentration than the temperature.

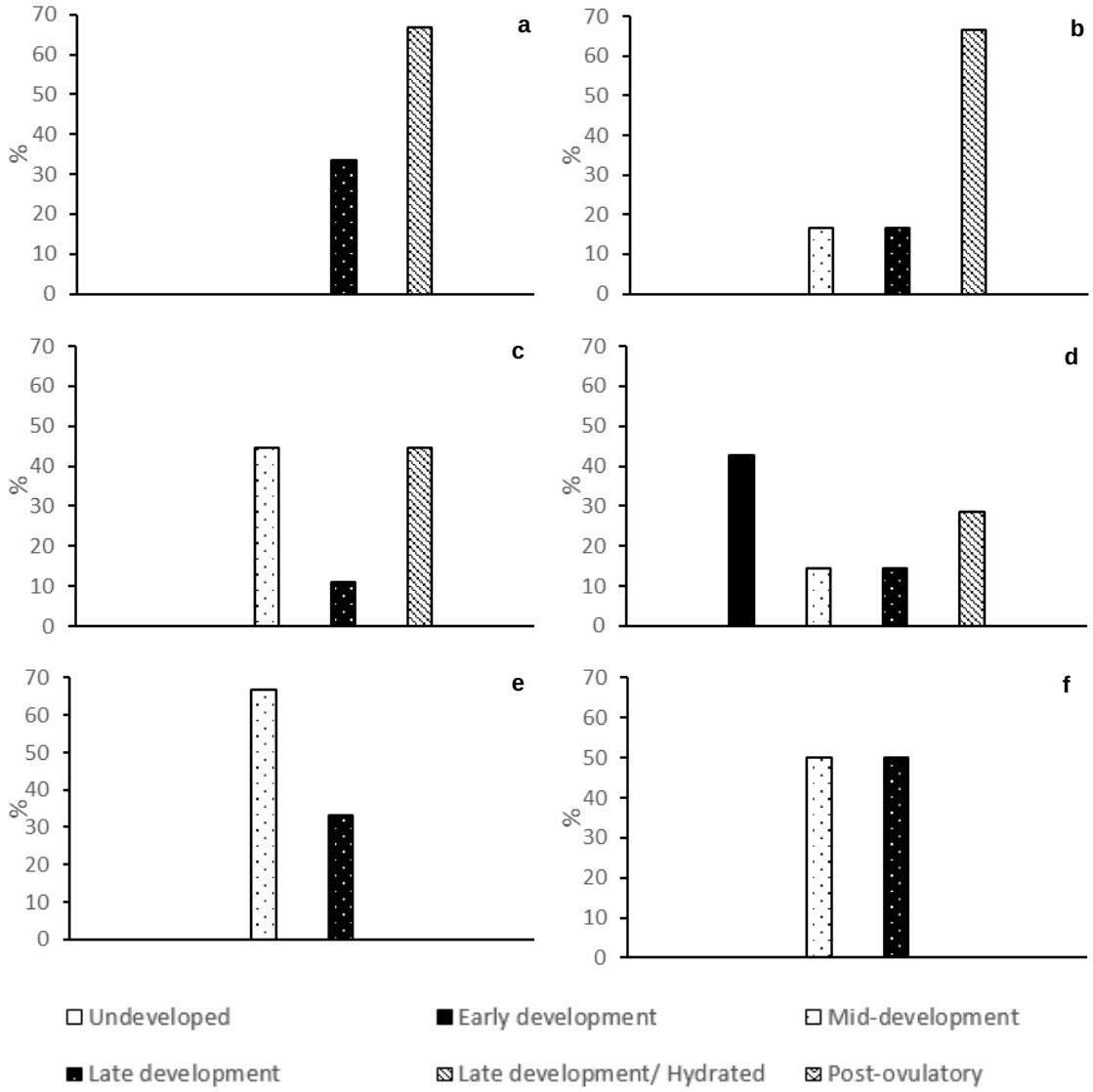

Figure 9 - Female gonadal maturation stages (represented in % of occurrence) in the pre-reproduction phase (day 21). **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC, **b)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC, **c)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC, **d)** L1T30 – LNG (10 ngL^{1}) at 30 ºC, **e)** L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, **f)** L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

Regarding males, we found testes varying from stages 1 to 3 (Figure 9), but any in stages 0 (undeveloped) or 4 (spent).

When grouping males by their gonadal maturity, the grading analysis allowed us to observe the overall predominant stage is the mid-spermatogenic (Figure 10). In line with this tendency, no significant differences for the LNG concentration and temperature were found (2-way ANOVA on ranks, $p > 0.05$). Yet, this analysis should not be viewed as definitive due to low number of fish analysed so far in some of the assayed conditions.

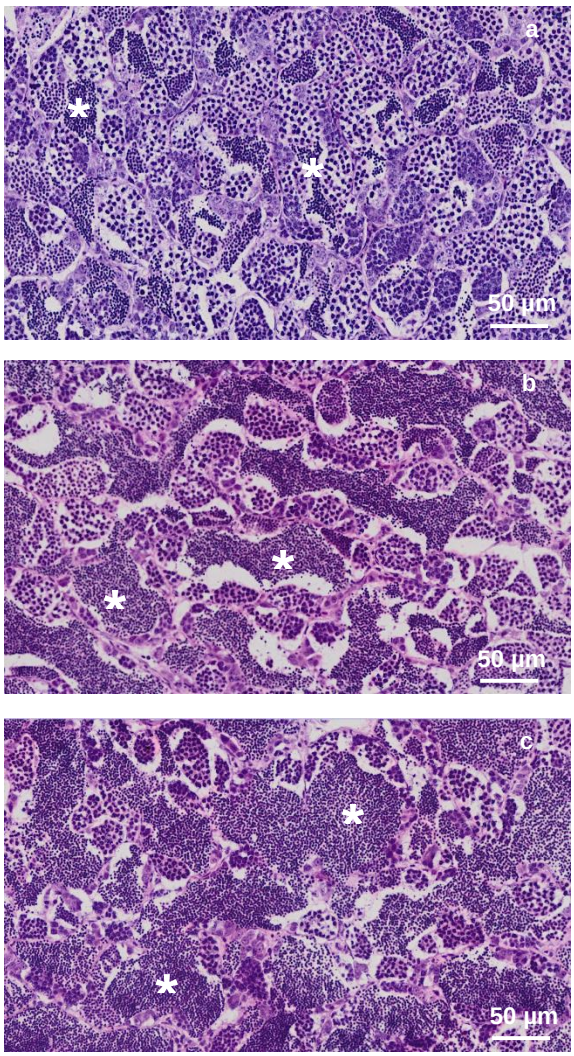

Figure 10 - Representative general histological aspects of the three stages of male grading. **a)** Stage 1 (early spermatogenic) from CtT30 – solvent control (0.01 % ethanol) at 30 ºC; **b)** Stage 2 (mid-spermatogenic) from L1T27 – LNG (10 ngL^{-1}) at 27 ºC; **c)** Stage 3 (late spermatogenic) from CtT30 – solvent control (0.01 % ethanol) at 30 ºC. Note the raising mass of mature sperm from **a** to **c** (asterisks).

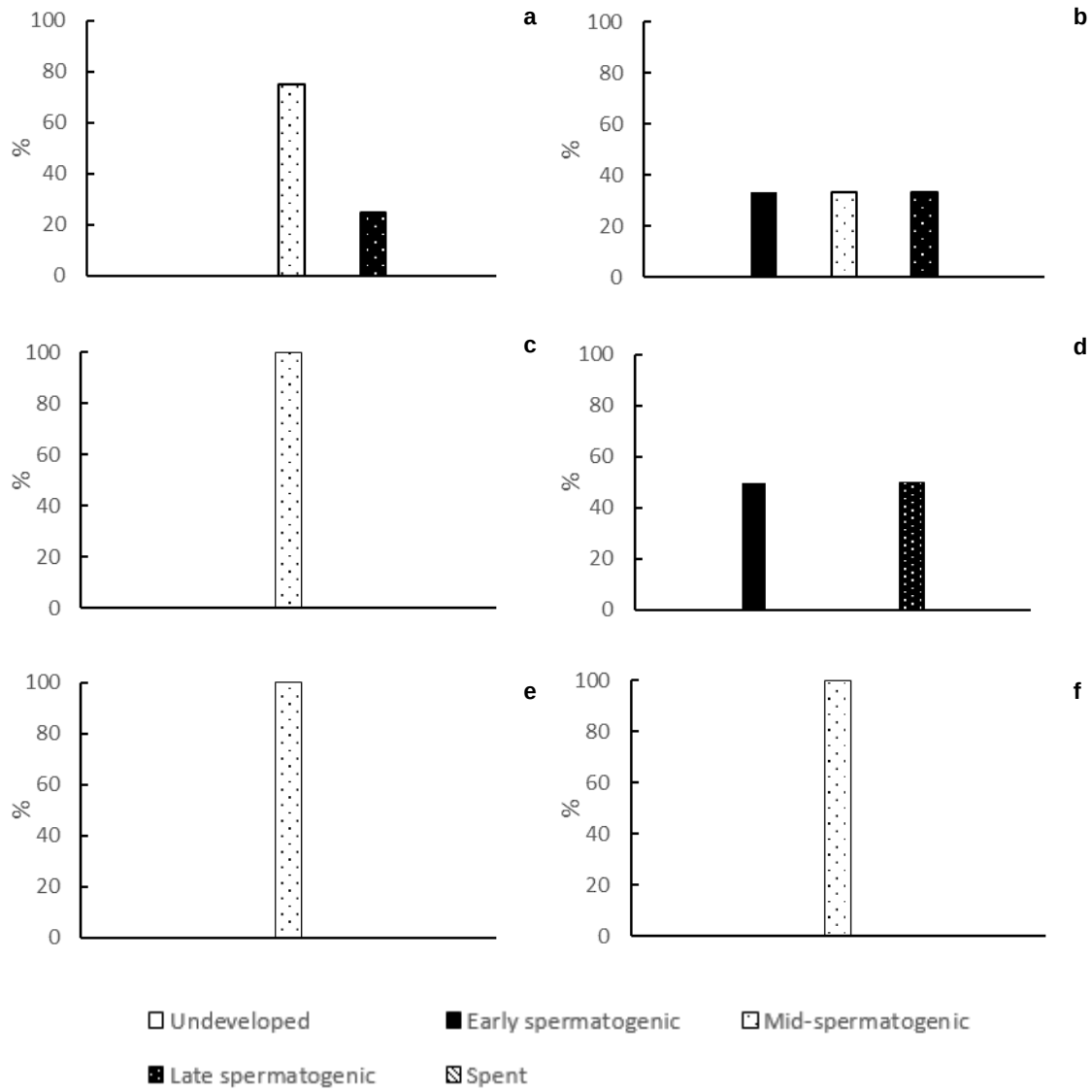

Figure 11 - Male gonadal maturation stages (represented in % of occurrence) in the pre-reproduction phase (day 21). **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC, **b)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC, **c)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC, **d)** L1T30 – LNG (10 ngL^{1}) at 30 ºC, **e)** L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, **f)** L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

3.5.2. Post-reproduction

Regarding the grading analysis of the ovaries in the post-reproduction phase, it is possible to observe that the percentage of females presenting less mature gonads increased when exposed to LNG (Figure 11c) compared to those under control conditions (Figure 11a, b). Also, higher temperature led to less developed gonads (Figure 11). Significant differences existed for the stages (1-way ANOVA on ranks, $F_{(2, 11)} = 5.6437$, $p = 0.0206$) among groups, showing that the treatment has an influence on the development of the female gonads after the reproduction.

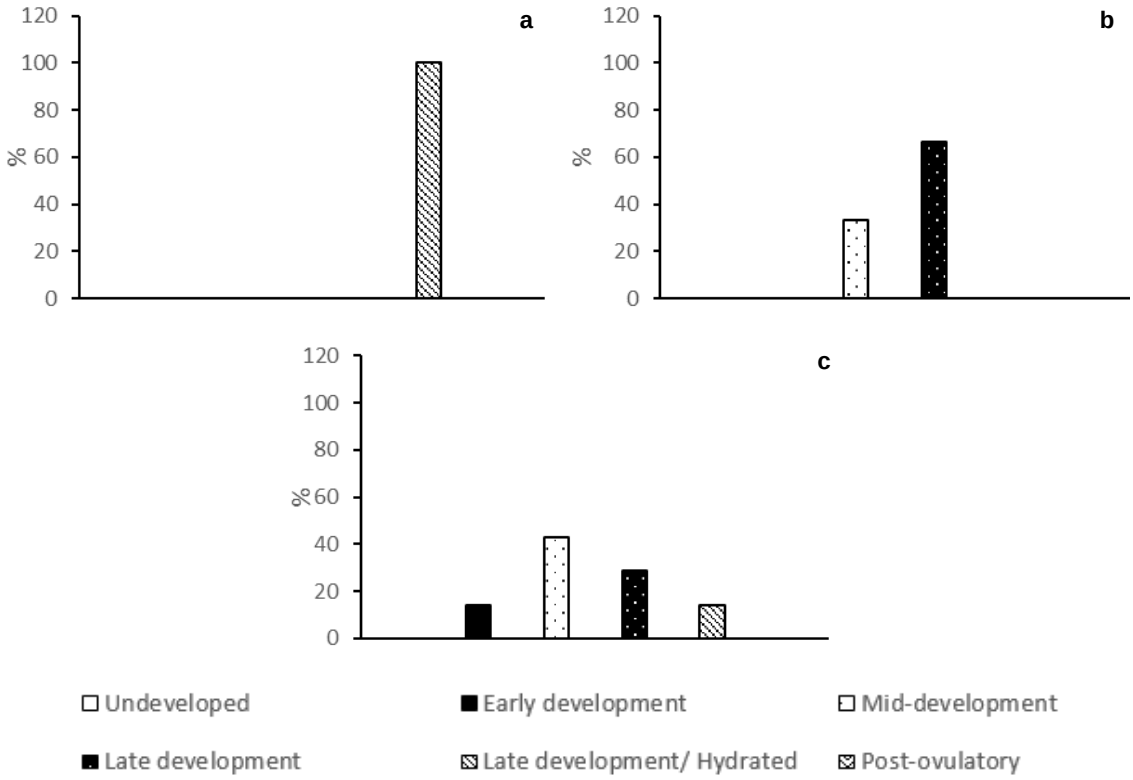

Figure 12 - Female gonadal maturation stages (represented in % of occurrence) in the post-reproduction phase (48 h after reproduction). **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC, **b)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC, **c)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC.

Regarding the grading analysis of the testis in the post-reproduction phase, at a first glance the percentage of males presenting less mature gonads seems to increase when exposed to LNG (Figure 12c) compared to those under control conditions (Figure 12a, b). However, eventually due to the current still low statistical power, no significant differences of the testis maturation extent depending on either LNG and/or temperature were found (1-way ANOVA on ranks, $p > 0.05$).

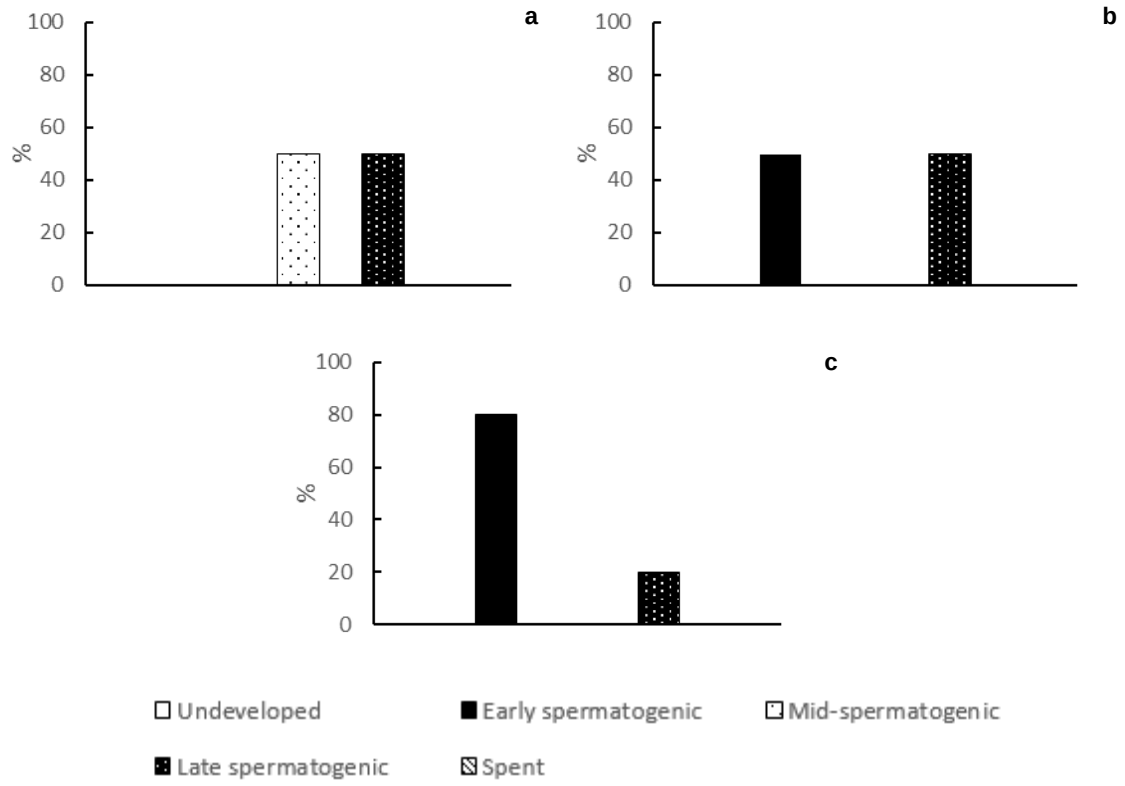

Figure 13 - Male gonadal maturation stages (represented in % of occurrence) in the post-reproduction phase (48 h after reproduction). **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC, **b)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC, **c)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC.

3.6. Stereological analysis

3.6.1. Pre-reproduction

For the females, it is clear that with the increase of LNG concentrations there was an increment in the relative volume density of early vitellogenic oocytes in detriment of late/mature ones (Figure 13). The temperature seemed to have a little effect on the volumetric distribution of the structural components of the gonad (Figure 14). Comparing each development stage for the different treatments we found significant differences for the LNG concentrations for two main compartments: early vitellogenic oocyte (2-way ANOVA on ranks, $F_{(2,39)} = 14.8813$, $p = 0.000016$) and the late/mature oocyte (2-way ANOVA on ranks, $F_{(2,39)} = 3.9775$, $p = 0.026790$). For the temperature, no significant differences were observed ($p > 0.05$). The other structural components did not show significant differences among treatments.

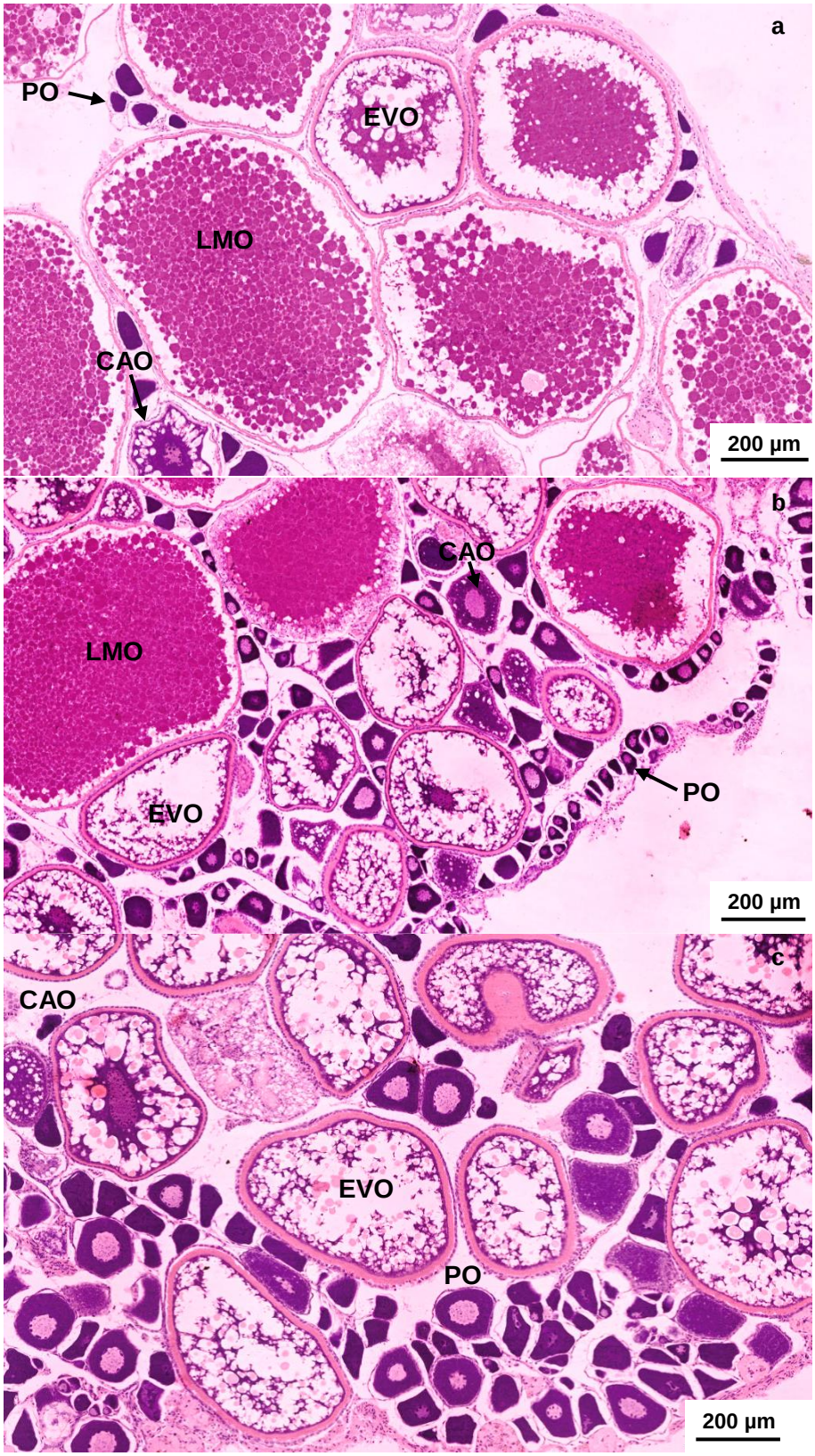

Figure 14 - Representative stereological analysis in pre-reproduction phase. **a)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC; **b)** L1T30 – LNG (10 ngL^{-1}) at 30 ºC; **c)** L2T30 – LNG (1000 ngL^{-1}) at 30 ºC. AO – Atretic oocyte; CAO – cortical-alveolar oocyte; EVO – early vitellogenic oocyte; LMO – late/mature oocyte; PO - primary oocyte.

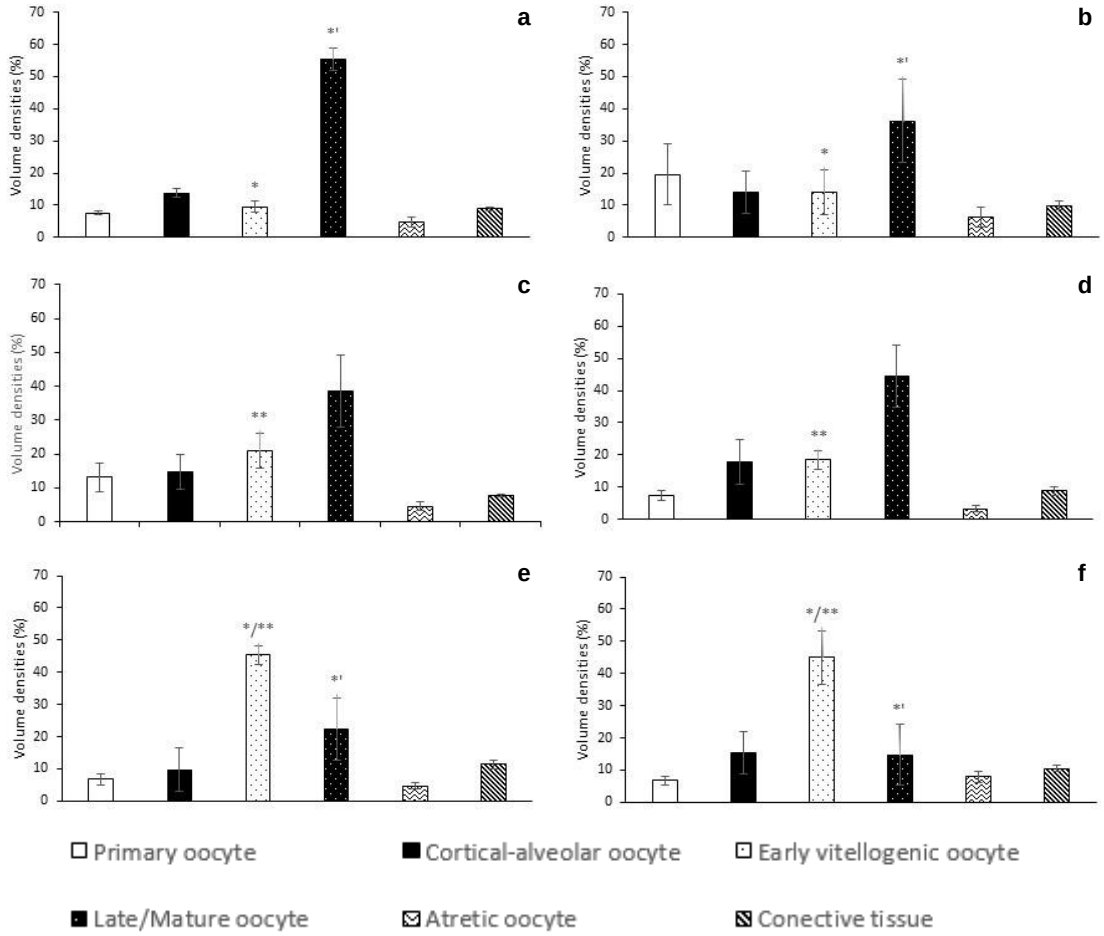

Figure 15 - Volume densities (V_V), in %, of each structural components of the ovarian tissue of female zebrafish in the pre-reproduction phase (day 21). Values represent mean ± SD. Significant differences between control and L2 are indicated with an * and *', for the early vitellogenic oocyte and late/mature oocyte, respectively. Significant differences between L1 and L2 for the early vitellogenic oocyte are marked with **. **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC, **b)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC, **c)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC, **d)** L1T30 – LNG (10 ngL^{-1}) at 30 ºC, **e)** L2T27 – LNG (1000 ngL^{-1}) at 27 ºC, **f)** L2T30 – LNG (1000 ngL^{-1}) at 30 ºC.

3.6.2. Post-reproduction

In the post-reproduction phase, the pattern was similar to that of pre-reproduction. Descriptive statistics is suggestive of a slight decrease of late/mature oocyte and a slight increase of early vitellogenic oocyte, from control to LNG exposure. As referred before, no data were collected for the L1T30, L2T27 and L2T30 treatments, because the reproduction did not occur in these treatments. Therefore, a 1-way ANOVA on ranks was performed to test for differences among treatments, however, the graphical tendencies do not correspond to significant differences (1-way ANOVA on ranks, $p > 0.05$).

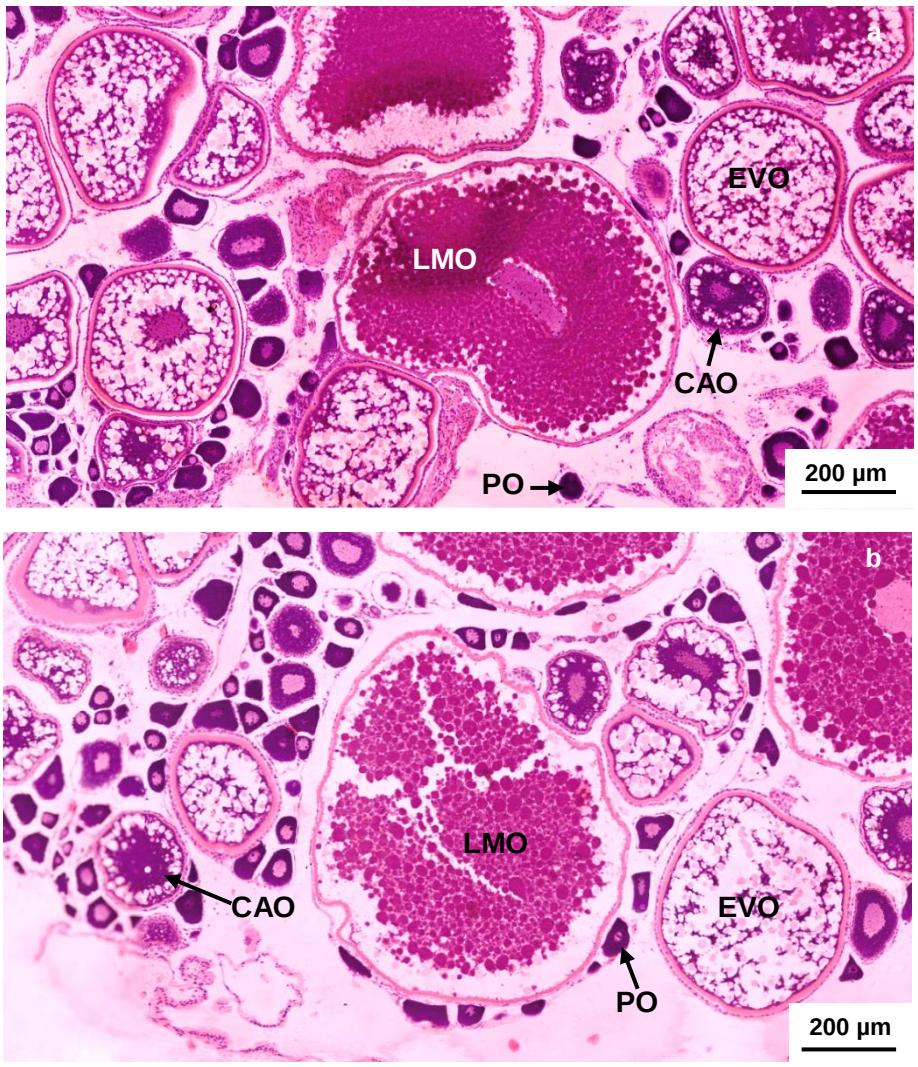

Figure 2 - Representative stereological analysis in post-reproduction phase. **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC; **b)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC. AO – Atretic oocyte; CAO – cortical-alveolar oocyte, EVO – early vitellogenic oocyte; LMO – late/mature oocyte; PO - primary oocyte.

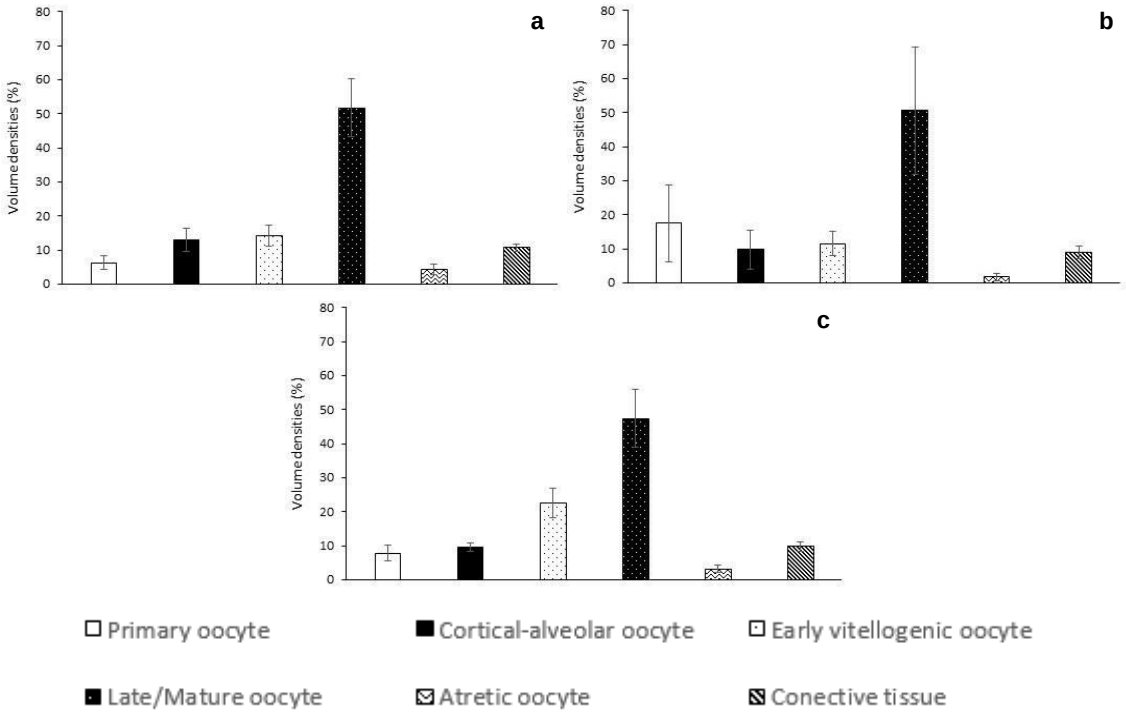

Figure 37 - Volume densities (Vv) in %, of each structural components of the ovarian tissue of female zebrafish in the post-reproduction phase (48 h after reproduction). Values represent mean ± SD. **a)** CtT27 – solvent control (0.01 % ethanol) at 27 ºC, **b)** CtT30 – solvent control (0.01 % ethanol) at 30 ºC, **c)** L1T27 – LNG (10 ngL^{-1}) at 27 ºC.

Chapter IV: Discussion

4. DISCUSSION

Worldwide, the global average temperature as well as the consumption of synthetic hormones is increasing and tend to rise more and faster in the near future (Christen et al., 2010; Miranda et al., 2013). The increase of temperature and the discharge of hormones are affecting the water bodies and consequently the quality of the aquatic ecosystems and their biodiversity (Maack and Segner, 2004; Noyes et al., 2009). In this study, it was hypothesised that the combination of both stress factors (climate and pollution by an endocrine disruptor) would bring an extra value and novelty to the work, since the majority of the studies just focus in isolated stressors (climate change or pollution, separately). According to this study, the fitness of zebrafish (as accessed by the Fulton's condition index) did not reveal any significant effects of both temperature and LNG, isolated or in combination. This can be explained based mainly on the exposure period, which could be too short to evidence such biometric differences. Indeed, the condition factor is better established for monitoring long-term impacts, and even is such cases there are restrictions of application (Teubner et al., 2015). Contrarily to our results, other studies, like Van den Belt et al. (2003) have already demonstrated a decline in the Fulton's K of the zebrafish embryos when exposed to 25 ngL^{-1} of 17*α*-ethynylestradiol for 3 months' post-fertilization. Also, Ensenbach and Nagel (1997) found a significant reduction in the Fulton's K of the fishes after 6 months of exposure to the mixture of 3,4-dichloroaniline (2 μgL^{-1}) and lindane (40 μgL^{-1}).

Concerning the GSI, despite it was observed a slight decline on its pattern with the increase of temperature and LNG concentration, no significant differences were observed. This in accordance with a previous study done by Madureira et al. (2011), in which adult zebrafish exposed to various pharmaceuticals, such as carbamazepine, fenofibrate, propranolol hydrochlorine, sulfamethoxazole and trimethoprim for 21 days did not evidence any effects on the GSI. However, Panter et al. (1998) studied the effects of 17β-oestradiol (320 and 1000 ngL^{-1}) and oestrone (318 ngL^{-1}) on plasma vitellogenin levels and gonad weight of the male fathead minnow (*Pimephales promelas*) for 21 days and found significant differences on the GSI, being the effects of 17β-oestradiol more pronounced that the effects of oestrone. This study showed that GSI was significantly affected by these oestrogens, being the testicular growth partially or totally inhibited during the 21 days of exposure (Panter et al., 1998). Despite, the GSI is frequently used as an indicator to evaluate the impact of some stressors, like contaminants, on the reproductive system of several species, in the present study this index was not able to reflect the significant changes observed within the ovary. So, this means that it should be interpreted

with some caution and always in association with other parameters (e.g. gonads histopathology) that can complement the analysis.

Regarding fecundity, this is another important reproductive parameter used in several studies (Brion et al., 2004; Maack and Segner, 2004; Xu et al., 2008; Chang et al., 2013). In the present study, zebrafish fecundity (decline on the mean number of eggs laid with increase of contaminant) was negatively affected by the increase of both levonorgestrel concentrations and temperature, with the hormone having a greater impact than temperature. According to our results, the two factors seemed to have combinatory (potentiation) effects, with temperature exacerbating the negative impact of the hormone. This was reflected on the absence of reproduction for the treatments exposed to the highest LNG concentrations (L2T27 and L2T30) and the lowest concentration at higher temperature (L1T30). This is in line with the finding that the synthetic progestin megestrol acetate (at 666 ngL^{-1}) reduced the cumulative egg production of zebrafish exposed to that hormone for 14 days (Han et al., 2014). Despite the biochemical mechanisms of action of progestins and oestrogens differ, impacts on fecundity may be derived from both types of compounds. In this view, our study is in part in accordance with a study from Xu et al. (2008), in which the zebrafish fecundity was also negatively affected by exposure to 17α-ethinylestradiol (EE_2: 10 ngL^{-1}) for 3 months. In addition, Chang et al. (2013) studied the effects of the weakly xenoestrogen butachlor (50 and 100 μgL^{-1}) on zebrafish reproduction for 30 days of exposure and found a significant decline on fecundity of treated groups compared to the control. It is of note that other pollutants that do not bind to sex-steroid receptors can affect, by various mechanisms, fecundity losses. For instance, Ansari and Ansari (2011), also found negative effects on zebrafish fecundity when exposed to four different sub-lethal concentrations of the organophosphate dimethoate (from 24.68 to 38.09 μgL^{-1}) for 96 hours; a compound that does not bind oestrogen receptors (Zheng et al., 2002) but rather acts by disruption of the pituitary-gonadal axis (Verma and Mohanty, 2009). In addition, Chang et al. (2013) studied the effects of butachlor (50 and 100 μgL^{-1}) on zebrafish reproduction for 30 days of exposure and found a significant decline on fecundity of treated groups compared to the control. Actually, the later mechanism is suspected to underlie negative effects of short term-exposures of the progestin megestrol on zebrafish reproduction (Han et al., 2014).

Besides fecundity, other reproductive parameters were studied such as the viability, fertility and survival rates. The viability rate is frequently used to understand the effects of different factors on the fish reproduction and is considered one of the most important endpoints in reproduction studies (Brooks et al., 1997; Kime and Nash, 1999; Soares et al., 2009). In the present study, we concluded that the viability rate (for the

treatments that reproduced) was not negatively affected by the temperature and the LNG. In fact, the treatments exposed to control warming (CtT30) and low LNG concentration at ambient temperature (L1T27) did not present a significant decline in the viability rate compared to the control ambient temperature (CtT27). Since the other treatments did not reproduce, the viability rate could not be analysed. These results are in part in agreement with those of Lin and Janz (2006). These authors studied the effects of a binary mixture of the xenoestrogen nonylphenol (NP) and 17α-ethinylestradiol (EE_2) on zebrafish reproductive capacity for 60 days. They showed no significant effects for the groups treated with low concentrations of the mixture (10 and 100 μgL^{-1} NP, 1 ngL^{-1} EE_2 + 10 μgL^{-1} NP, 10 ngL^{-1} EE_2 + 10 μgL^{-1}), nevertheless the opposite scenario occurred for the highest concentrations of the mixture (1 and 10 ngL^{-1} EE_2, 1 ngL^{-1} + 100 μgL^{-1}, 10 ngL^{-1} + 100 μgL^{-1}) where a significant reduction on the eggs viability was confirmed compared to the control (Lin and Janz, 2006). A similar result was also observed by Van der Ven et al. (2006) that studied the effects of the antidepressant mianserin (0, 25 and 250 μgL^{-1}) in egg production, fertilization and hatching in zebrafish for 14 days'. In this work a concentration-related reduction in egg viability was noted, occurring a significant decrease of viability on the highest concentration compared to the control (Van der Ven et al., 2006).

Another important parameter to be studied is the fertility rate, which is usually used to evaluate the effects of several stressors on the reproductive success of distinct species (Zohar and Mylonas, 2001; Coward et al., 2002; Mills and Chichester, 2005). In the present study, the fertility rate presented a similar pattern to the viability rate and despite we could observe a slight decline in fertility rate in the treatment exposed to low LNG concentration (at the lowest ambient temperature), there was not a statistically significant effect of the hormone. Van den Belt et al. (2001) studied the impacts of ethinylestradiol (EE_2: 5, 10, 25 and 50 ngL^{-1}) and of the weak xenoestrogen 4t-octylphenol (OP: 12.5, 25, 50 and 100 μgL^{-1}) on zebrafish reproduction for 3 weeks, at a temperature varying from 25 to 29 ºC. In accordance with our findings, their results have shown a trend for an adverse impact on fertility (EE2: 10 and 25 ngL^{-1} and OP: 25, 50 and 100 μgL^{-1}), although no statistically significant effects were found for none of the compounds studied. On the other hand, Schultz et al. (2003) studied the effects of 17α-ethinylestradiol (EE_2: 10, 100 and 1000 ngL^{-1}) on the reproduction of male rainbow trout (*Oncorhynchus mykiss*) for 62 days. The results indicated that fertility decreased significantly in the groups treated with EE_2 compared to the control. In addition to important differences among fish species as to sensitivity for oestrogens (Miyagawa et al., 2014), probably the longer exposure time associated to higher EE_2 concentrations led to higher impact on fertility than on the study

of Van den Belt et al. (2001). In the present study, since we were restricted to the treatments that reproduced and the lower LNG concentration corresponded to a lower environmental concentration, maybe that is why the results were not so evident. Probably, if the zebrafish is able to reproduce under higher concentrations probably the fertility rate would be negatively affected. Indeed, this is what was observed when fathead minnow (*Pimephales promelas*) were under 21-day exposure to progesterone (P4: at 10, 100 and 1000 ngL^{-1}), which caused dose-dependent reductions in female fertility and fecundity (DeQuattro et al., 2012).

Other parameter that was studied was the survival rate. According to our results no significant effects of LNG and temperature were recorded on eggs survival, despite we could observe a slight decline on survival rate when exposed to low LNG concentration (the simulated normal ambient temperature). Ensenbach and Nagel (1997) studied the effects of a mixture of the herbicide metabolite 3,4-dichloroaniline (100 μL^{-1}) and the organochlorine lindane (40 μL^{-1}) in zebrafish reproduction for 6 months and no significant effects were found for survival rate after exposure time compared to the control. Lin and Janz (2006) studied the effects of a binary mixture of nonylphenol (NP) and 17α-ethinylestradiol (EE_2) on the reproductive capacity of zebrafish for 60 days and the results have shown no significant effects on survival rate among treatments. As to assays with progestogens, 21-day exposures either to the natural progesterone or to megestrol, respectively in fathead minnow (DeQuattro et al., 2012) and zebrafish (Han et al., 2014) produced noteworthy impacts neither in hatching rate nor in embryonic development. These results are in agreement with our findings, however, since in the present study the organisms exposed to the maximum concentration did not reproduce, we cannot be conclusive about the effect of higher LNG concentrations on the embryos survival.

Finally, the embryos eclosion rate was also studied and according to our results, this parameter seems to be entirely related to temperature. So, the higher the temperature the faster the eclosion rate. Also, we could observe a decline in the eclosion rate of the embryos exposed to low LNG concentration compared to the controls. The close relation between temperature and the eclosion rate can be related to the metabolic theory. According to this theory an increase in temperature will generally, lead to an increase in metabolic rate that triggers many processes, like growth, consumption, reproduction and embryonic development (Brown et al., 2004; O'Connor et al., 2009). Higher temperatures increase kinetic energy in cells by speeding up the molecules involved in chemical reactions, leading to an acceleration in eggs eclosion (Brown et al., 2004). Irrespective of the fact that temperature is a vital factor on the embryos eclosion time, since it can accelerate the embryonic development (Brown et al., 2004), we could also observe herein

a slight negative effect of the LNG on the eclosion rates. Impacts of EDC on the eclosion rate of zebrafish embryos have been verified for different types of compounds. For example, Hill and Janz (2003) that studied the effects of the weak oestrogen 4-nonylphenol (NP: 100 μgL^{-1}) and of 17α-ethinylestradiol (EE_2: 10 ngL^{-1}) on sex ratio and on breeding success, for 120 days post-hatching (60 days of exposure and 60 days of depuration), found and a significant reduction in eclosion rates.

For better understanding the results observed on the zebrafish reproduction, it was also evaluated the pattern of gonads maturation, through two complementary analyses, first by semiquantitative grading the global ovary status, and subsequently by a refined stereological analysis. In this study, due to time limitations, it was only considered the female gonads. Additionally, we did not adequately recover all the testes for all males, which impaired us to have a sufficient number of males for all the experimental groups. Therefore, the still scar histological data about males is to be viewed as an exploratory indication that will be pursued in the future. Indeed, there are already evidences that male fish can be impacted by progestins, including LNG and drospirenone, for example in the form of germinal epithelium thickening and spermiation reduction, as shown in fathead minnow at doses < 30 ngL^{-1} and exposures for 21 days (Zeilinger et al., 2009).

According to the grading analyses, it was observed a decline in the percentage of females with matured gonads after exposure to LNG. This effect was exacerbated by the increase of temperature. Van der Ven et al. (2003) studied the effects of exposure to the estrogen (E_2) and the nonaromatizable androgen 17-methyldihydrotestosterone (MDHT) and observed that the ovary of E_2-exposed animals lacked advanced maturation stages. After exposure to 100 μgL^{-1} MDHT for 3 weeks, the ovary showed a significant shift from vitellogenic to previtellogenic oocytes, so the maturation stages reduce during the exposure time (Van der Ven et al., 2003). It is opportune to mention that, like MDHT, LNG have androgenic actions via the androgen receptor, including in zebrafish, as demonstrated by the strong masculinization effect (viz. 100% sex reversal towards males) detected after exposure of embryos to sexually matured adults (142 dph), at concentration of 10, 33 and 100 ng L^{-1} (Hua et al., 2015). Other impacts of LNG (at 26.6 ngL^{-1}) on the ovary maturation status, after exposures of up to 21 days, were also observed in the fathead minnow, with changes in frequency of oocyte types and abnormal retention of mature ones (Zeilinger et al., 2009). The latter is a scenario that despite indicating disruption is not similar in detail to that found here in zebrafish. In addition to divergences inherent to various experimental details, different disruptions on females of diverse fish species suggest that LNG has species-specific impacts, and certainly unequal sensitives for the same progestins, even at the same range of doses.

Through the stereological analysis, it was possible to observe that the female gonads (pre-reproduction) from control (ambient temperature) presented almost 60 % of the ovarian tissue occupied by late/mature oocytes. This value tended to decline with the highest LNG concentration in favour of a greater compartment of early vitellogenic oocytes. In addition, the increase of temperature seemed to have exacerbated the effect of the hormone. Thus, after 21 days of exposure, female fish exposed to the highest LNG concentration (1000 ngL^{-1}) at both temperatures (27º and 30 ºC) exhibited mainly immature oocytes, with an even lower percentage of mature oocytes at 30 ºC. In the post-reproduction phase, it was possible to observe a similar trend despite the treatments from L1T30, L2T27 and L2T30 were not represented, due to the absence of reproduction. To our best knowledge, we do not have yet stereological studies on the fine effects of progestins in fish ovaries to compare with our data. However, two other studies of stereological impacts on zebrafish ovary after 21 days of exposures, either to a panel of pharmaceutical products (Madureira et al., 2011) or to xenoestrogens (a mixture of 11 compounds or EE_2 singly) (Silva et al., 2012), evidenced decreases in the volumes of the most mature oocyte-follicle stages, with concurrent increases of less mature follicular stages. For the latter study, with estrogenic EDCs, the results showed a decrease of relative volumes of the more advanced maturation stages of oocytes with EE_2; the same kind of result was found for the estrogenic mixture, but with less impact. Overall, all the data suggest that the maturation of (at least) the zebrafish ovary can be impacted in the same histopathological way (i.e., reduction of the pool of more mature oocytes) by compounds with various modes of action for acting as gonadal-disrupters.

Based on the grading and stereological analyses of the ovary it was possible to better understand the results obtained at the reproduction level, since, in general, less mature gonads may ultimately lead to less fertile or eventually infertile females (no laid eggs) if final maturation fails. The only exception was observed for the females from L1T30, which presented a maturation level very similar to those from L1T27, however, they did not reproduce. Maybe this can be related with a stress response that probably supress the reproduction. Even if the gonads of L1T27 and L1T30 were in the same developmental stage, the L1T30 fishes were more stressed, because they were being exposed to a higher temperature. The central nervous system (CNS) regulates all the stress responses through neuro-endocrine pathways, and in this specific case probably the stress response led to suppression of reproductive functions, which is perhaps an adaptive response to conserve energy during difficulties (Rabin et al., 1988). However, we stress that this study was within the advocated range of temperature of 27-30 ºC for handling zebrafish in laboratory, actually shorter than that in nature (Lawrence, 2007).

Concluding, the exposure to a high LNG concentration for 21 days appears to supress the normal gonad maturation, which can be exacerbated by the increase of temperature. In fact, it is possible to conclude that the hormone LNG had a higher negative impact on the gonads maturation and reproduction than the temperature as isolated stressors. This means that in a future scenario of global warming associated to the progestins pollution, zebrafish, and other fish species alike, and most likely other aquatic organisms too, can face increased toxicological risks since their reproductive success can be endangered. This possibility should merit efforts for studying it in detail.

Chapter V: Conclusions

5. CONCLUSIONS

Global climate change is affecting multiple ecosystems worldwide. Temperature is one of the most relevant environmental variables that controls the overall functioning of aquatic organisms. Recent studies have proven that near future warming scenario predicted for 2100 may trigger serious implications on aquatic ecosystems, namely freshwater systems. Besides climate change, the increasing consumption of pharmaceuticals, like synthetic progestins, can also constitute a stress factor for the aquatic organisms. Most of the literature is focused on the evaluation of the effects of climate drivers on the structure and functioning of ecosystems, however, there is a great lack of information regarding the interactive effects of climate change and chemical pollution, including in what regards endocrine disruptor molecules, such as progestins.

The present study is one of the few works in the literature that deals with the interaction of the dual stresses of climate change and chemical exposures. According to our findings, the synthetic progestin LNG revealed to have a higher negative impact on gonadal (at least in ovary) maturation and reproduction than the temperature. However, the temperature seems to exacerbate the effect of LNG, which means that they can act jointly to elicit potentiated effects. Therefore, the combination of those factors can lead to an increase in the percentage of subfertile/infertile zebrafish females, and consequently to lower reproduction levels.

In a scenario of global warming associated to the global trend of pharmaceuticals consumption, our data support the notion that it is expectable that aquatic communities face increased risks when such warming concomitantly occurs with other factors that are known to disturb reproduction. In accord, such kind of concomitancy must be prevented.

As future perspectives, it is a priority to make the evaluation of all the assayed conditions in the males too. Additionally, it is pertinent to evaluate the combined effects of climate change with other synthetic progestins of various classes, in zebrafish but also in other aquatic organisms, including both freshwater and marine species. In addition, it would be very relevant to observe the effects of multiple stressors on the following generations and evaluate if they would be more resistant than the progenitors or not. Therefore, further research on this topic is necessary to understand better the interactive effects of the dual stresses on the functioning of aquatic ecosystems. In this way, it is possible to contribute to a better management and conservation of those ecosystems.

Chapter VI: References

6. REFERENCES

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Chapter VII: Appendixes

APPENDIX I

Tissue processing in Leica 1020

1. Ethanol 70% (Proclínica, Portugal) – 1 hour
2. Ethanol 95% (Proclínica, Portugal) - 1 hour
3. Ethanol 95% (Proclínica, Portugal) - 1 hour
4. Ethanol 100% (Proclínica, Portugal) - 1 hour
5. Ethanol 100% (Proclínica, Portugal) - 1 hour
6. Ethanol 100% (Proclínica, Portugal) - 1 hour
7. Ethanol and xylene (50:50, v/v) – 1 hour
8. Xylene (VWR, France) – 1 hour
9. Xylene (VWR, France) – 1 hour
10. Paraffin (Thermo Fisher Scientific, EUA) – 1 hour
11. Paraffin (Thermo Fisher Scientific, EUA) – 1 hour

APPENDIX II

Haematoxylin-eosin staining

Dewaxing

1. Xylene (VWR, France) – 10 minutes
2. Xylene (VWR, France) – 10 minutes

Hydration

3. Absolute etanol (Proclínica, Portugal) – 5 minutes
4. Ethanol 95% (Proclínica, Portugal) - 5 minutes
5. Ethanol 70% (Proclínica, Portugal) - 5 minutes
6. Running tap water – 5 minutes

Staining

7. Mayer's haematoxylin (Merck, Germany) – 2 minutes
8. Wash in running water – 5 minutes
9. Quick differentiation in ethanol-acid (0,5% HCl in ethanol 80%)
10. Wash in running tap water – 5 minutes
11. Eosin 1% (Merck, Germany) - 1 minute
12. Wash quickly in running water

Dehydration

13. Absolute ethanol I (Proclínica, Portugal) – several dips
14. Absolute ethanol II (Proclínica, Portugal) - several dips
15. Absolute ethanol III (Proclínica, Portugal) - several dips

Diafanization

16. Xylene (VWR, France)
17. Xylene (VWR, France)

Mount using a resin mounting media for definitive preparations Coverquick 2000 (VWR, France)

APPENDIX III

Technovit 7100 Procedure

Dehydration

1. Ethanol 90% (Proclínica, Portugal) - 1h
2. Ethanol 96% (Proclínica, Portugal) - 1h
3. Ethanol 100% (Proclínica, Portugal) - 1h
4. Ethanol 100% (Proclínica, Portugal) - 1h

Pre infiltration

5. Infiltration solution (ready for several procedures): 100 mL Technovit + 1 g hardener I – shake for 10 minutes
   Misture 1:1 – Infiltration solution:ethanol 100% (necessary amount for the number of samples to process – 1 mL for piece) (2-4h)

Infiltration

6. Overnight infiltration (1 g of hardener is dissolved in 100 mL of Technovit agitate for 10 minutes) (1-12h)

Polimerization

7. Mixture 15:1 (15 mL of infiltration solution for 1 mL of hardener II)

Inclusion

8. Leave at 23°C (room temperature) for 1h and leave overnight at 37°C

APPENDIX IV

Methacrylate resin haematoxylin-eosin staining

1. Place the slide with Mayer's haematoxylin (Merck, Germany) in a hot plate at 60 ºC (20-30 min): do not let haematoxylin dry
2. Wash carefully with distilled water
3. Cover the slides with eosin (Merck, Germany) in a hot plate (7-10 min)
4. Let the slides completely dry
5. Wash carefully with distilled water
6. Assemble the slide with mounting media

APPENDIX V

Progestin Extraction Protocol

Preparation of water samples

1. Collection of the water samples, from experimental aquaria, into clean amber flasks, which should be immediately refrigerated at ± 4 °C
2. Filtration of the water samples (2 L) by 0.45 µm glass fibre filters under vacuum and wash the filter with 2 mL of CH_3OH
3. Acidification of the filtered waters with H_2SO_4 conc. (pH= 2)
4. Storage of water samples at ± 4 ºC in dark until further processing (max. 48 h)

Extraction and preconcentration protocol (Ribeiro et al., 2007), adapted for quantification of the synthetic progestin LNG by Ultra-High Pressure Liquid Chromatography (UPLC)

5. Conditioning the SPE 200 mg Oasis HLB cartridges adapted in an off-line SPE vacuum extraction device (Waters), at a flow rate of 1 mL min-1:
    a. 13 mL of CH_3OH:CH_2Cl_2 (50:50, v/v)
    b. 6 mL of CH_3OH
    c. 13 mL of ultrapure Milli-Q water
6. Load of the water samples, onto the SPE conditioned cartridges at a constant flow rate of 5 mL min^{-1}
7. Wash the SPE cartridges with 13 mL of ultrapure Milli-Q water, followed by 500 µL of CH_3OH
8. Dry the SPE cartridges at vacuum during 30 minutes

Final extraction and preconcentration of LNG from water samples

9. Creation of the system formed by the 200 mg Oasis-HLB cartridges coupled to 1 g Sep-Pak silica cartridges (Fig. 17) for the elution of LNG with 10 mL of (50:50, v/v)
10. The extracts were evaporated to dryness in a heating block at 40 °C under a gentle stream of nitrogen and stored at -20 °C
11. Before UPLC analysis the extracts were reconstituted in 20 µL of (C_2H_3N)

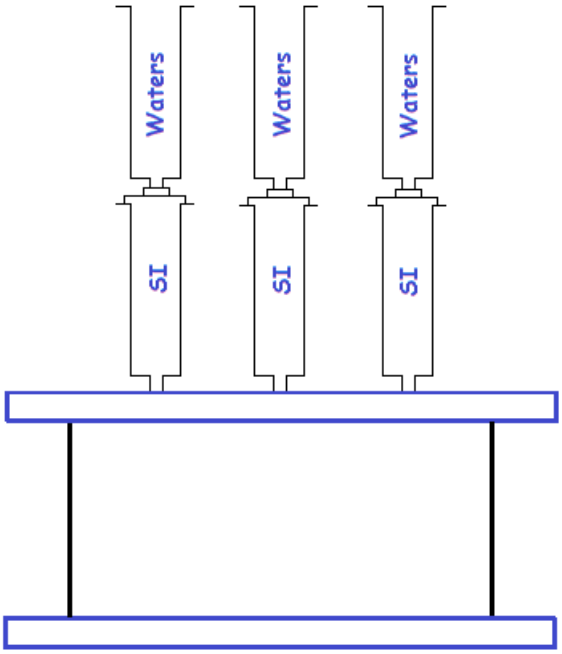

Figure 4 - Scheme of the system to progestin extract.